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PARAZITÉ ZPŮSOBUJÍCÍ PTAČÍ MALÁRII A JEJICH PŘENAŠEČI

AVIAN MALARIA PARASITES AND THEIR VECTORS

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Dizertační práce

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RNDr. Petr Synek

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Jsem prvním autorem tří publikací, které jsou hlavní součástí předkládané dizertační práce, u dvou dalších publikací jsem spoluautorem. Můj podíl na jednotlivých publikacích je popsán níže.

I) Synek P, Munclinger P, Albrecht T, Votýpka J (2013a). Avian haemosporidians in haematophagous insects in the Czech Republic. Parasitol Res 112: 839–845. Doi: 10.1007/s00436-012-3204-3

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II) Synek P, Albrecht T, Vinkler M, Schnitzer J, Votýpka J, Munclinger P (2013b). Haemosporidian parasites of a European passerine wintering in South Asia: diversity, mixed infections and effect on host condition. Parasitol Res 112: 1667–1677. Doi: 10.1007/s00436-013-3323-5

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III) Synek P, Popelková A, Munclinger P, Koubínová D, Šťastný K, Langerová I, Votýpka J (2016). Haemosporidian infections in the Tengmalm's Owl (*Aegolius funereus*) and potential insect vectors of their transmission. Parasitol Res 115: 291–298. Doi: 10.1007/s00436-015-4745-z

Podíl Petra Synka na publikaci: Design studie, odběr vzorků vektorů v terénu, laboratorní analýza (Nested PCR a další), získání sekvencí DNA, analýza dat, interpretace výsledků, příprava draftu a následné úpravy rukopisu.

Spoluautorské publikace:

IV) Svobodová J, Gabrielova B, Synek P, Maršík P, Vaněk T, Albrecht T, Vinkler M (2013). The health signalling of ornamental traits in the Grey Partridge (*Perdix perdix*). J Ornithol 154: 717–725. Doi: 10.1007/s10336-013-0936-5

Podíl Petra Synka na publikaci: Laboratorní analýza vzorků krve a detekce parazitů (Nested PCR a další), získání sekvencí DNA haemosporid, analýza parazitologických dat a jejich interpretace.

V) Hanel J, Doležalová J, Stehlíková Š, Modrý D, Chudoba J, Synek P, Votýpka J (2016). Blood parasites in northern goshawk (*Accipiter gentilis*) with an emphasis to *Leucocytozoon toddi*. Parasitol Res 115:263–270. Doi: 10.1007/s00436-015-4743-1

Podíl Petra Synka na publikaci: Laboratorní analýza vzorků krve a detekce ptačích haemosporid (Nested PCR a další), získání sekvencí DNA, analýza získaných dat a jejich interpretace.

Podpis školitele:

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Doc. Mgr. Pavel Munclinger, Ph.D.

OBSAH

ABSTRAKT

Parazity způsobující ptačí malárii řadíme mezi Haemosporida, která představují monofyletickou skupinu dixenních protist patřících do kmene Apicomplexa. Jejich nepohlavní množení probíhá v obratlovčím mezihostiteli a ke vzniku gamet a sporogonii dochází v krevsajícím dvoukřídlém hmyzu, který je tudíž konečným hostitelem těchto parazitů. U ptáku jako mezihostitelů se setkáváme s následujícími třemi rody těchto parazitů: *Plasmodium*, *Haemoproteus* a *Leucocytozoon*.

V disertační práci jsem se soustředil na Haemosporida volně žijících ptáků a na způsob jejich přenosu hmyzími vektory v přírodních populacích, což byla prozatím opomíjená oblast výzkumu zaměřeného na původce ptačí malárii. Výsledky byly získány jak tradičními metodami (vyšetření infekcí pomocí mikroskopování krevních roztěrů), tak především molekulárně biologickými metodami (detekcí parazitů pomocí nested PCR) založenými na práci s unikátními haplotypy jednotlivých linií haemosporid.

Cílem studie bylo určit okruh možných přenašečů ptačích haemosporid na území České republiky s přihlédnutím ke specificitě ptačích haemosporid v rámci těchto vektorů a popsat diverzitu ptačích haemosporid v populacích jejich ptačích mezihostitelů. K tomu jsme si vybrali čtyři různé druhy ptáků, patřících do čtyř řádů (pěvci – Passeriformes, sovy – Strigiformes, dravci – Accipitriformes a hrabaví – Galliformes).

Jako potencionální přenašeče krevních haemosporid na území České republiky jsme určili tiplíky druhů *Culicoides kibunensis*, *C. segnis* a *C. festivipennis*, komáry komplexu *Culex pipiens* a muchničky druhů *Eusimulium angustipes* a *Nevermannia vernum*. Nalezli jsme u nich celkem 12 linií (unikátních haplotypů) ptačích haemosporid, čtyři linie byly nové. V době vzniku našich prvních publikací se jednalo o jedny z úplně prvních publikací věnujících se komplexně vektorům a přenášeným prvokům (převážně tiplíkům jako přenašečům rodu *Haemoproteus* a muchničkám jako přenašečům rodu *Leucocytozoon*). Na tyto práce s rozvojem zavádění molekulárních metod do studia přenašečů haemosporid navázalo velké množství studií. Prevalence se v populacích ptačích hostitelů pohybovaly od 12 % u koroptví polních (*Perdix perdix*) po 86 % v populaci sýce rousného (*Aegolius funereus*). Detekovali jsme u nich celkem 27 linií (haplotypů) všech tří hlavních rodů ptačích haemosporid (*Plasmodium*, *Haemoproteus* a *Leucocytozoon*), z nichž 8 bylo zcela nových.

Klíčová slova:

Ptačí krevní parazité, Haemosporida, PCR detekce, ptačí malárie, choroby volně žijících živočichů, přenašeči, přenos infekce, krevsající hmyz, *Plasmodium*, *Haemoproteus*, *Leucocytozoon*

ABSTRACT

Parasites causing avian malaria belong to the group Haemosporida, which represents a monophyletic group of dixenic protists within Apicomplexa. Their asexual reproduction takes place in a vertebrate intermediate host, and the formation of gametes and sporogony occur in blood-sucking dipteran insects, which are the definitive hosts of these parasites. Three main genera (*Plasmodium*, *Haemoproteus* and *Leucocytozoon*) are found mostly in their avian hosts.

We focused on the Haemosporida of wild birds and their transmission by insect vectors in natural populations, which had previously been a neglected area. Our results were obtained both by traditional methods (investigation of infections by microscopy of blood smears) and mainly by molecular methods (e.g. nested PCR) centered around work with unique haplotypes of the haemosporid lineages. The aim of our work was to determine the range of possible insect vectors of avian haemosporidians in the territory of the Czech Republic, taking into account the specificity of the parasites within these vectors, and to describe the diversity of haemosporidians in the populations of their bird intermediate hosts. We chose four different species of birds from four orders (Passeriformes, Strigiformes, Accipitriformes, and Galliformes).

As potential vectors of avian blood haemosporidians in the Czech Republic, we identified the biting midges of the species *Culicoides kibunensis*, *C. segnis* and *C. festivipennis*, mosquitos of the *Culex pipiens* complex and black flies of the species *Eusimulium angustipes* and *Nevermannia vernum*. In the vectors we found a total of 12 haemosporid lineages; four of them were new. These results represented some of the very first publications dealing with avian malaria vectors using molecular methods (especially for biting midges as *Haemoproteus* vectors and for black flies as *Leucocytozoon* vectors). Later on, our results were followed by numerous further studies, corresponding to an expansion of molecular detection methods. Prevalence in the bird intermediate hosts ranged from 12 % in Grey Partridge (*Perdix perdix*) to 86 % in Tengmalm's owls (*Aegolius funereus*). We detected a total of 27 lineages (unique haplotypes) of all three major avian haemosporidians (*Plasmodium*, *Haemoproteus* and *Leucocytozoon*), of which 8 were completely new.

Keywords:
Avian blood parasites. Haemosporida. PCR detection. Avian malaria. Wildlife diseases. Vectors. Transmission. Blood-sucking insects, *Plasmodium*. *Haemoproteus*. *Leucocytozoon*.

ÚVOD

Parazité ze skupiny Haemosporida, způsobující onemocnění souhrnně nazývané jako ptačí malárie, tvoří monofyletickou skupinu dixenních protist z kmene Apicomplexa (Martinsen et al. 2008). Jejich merogonie a tvorba gametocytů se odehrává v mezihostiteli, typicky obratlovci a ke vzniku gamet a sporogonii dochází v krevsajícícm dvoukřídlém hmyzu, který je tudíž konečným hostitelem těchto parazitů (Valkiūnas 2005). U ptačích mezihostitelů se setkáváme nejčastěji se třemi rody těchto parazitů, konkrétně rodem *Plasmodium*, *Haemoproteus* a *Leucocytozoon* (Pacheco et al. 2017). V obecné rovině představují Haemosporida dlouhodobě jednu z nejlépe prozkoumaných skupin protist a to především proto, že sem patří původci lidské malárie – krvinkovky (*Plasmodium falciparum*, *P. vivax*, *P. ovale* a *P. malariae*) (Tangpukdee et al. 2009). Existují ale velké rozdíly v našich znalostech biologie různých taxonů a ekologických skupin těchto parazitů: velká většina studií se pochopitelně věnuje lidským plasmodiím, případně těm druhům plasmodií, které slouží jako modelové organismy ke studiu lidské malárie – tzn. nejčastěji tedy druhům z drobných hlodavců (Orfano et al. 2016; Haeberlein et al. 2017). Přestože jsou ptačí Haemosporida početně i ekologicky nejvýznamnější skupinou, věnuje se jim jen menší část studií. Navíc významná část poznatků o jejich biologii má svůj původ v době, kdy se ptačí malaričtí parazité rovněž používali jako model pro studium malárie lidské. Tento fakt vedl k vysokému zastoupení počtu studií pracujících s údaji získanými experimentálně v laboratorních podmínkách jako objasnění jejich životního cyklu, interakce s vektorem, vývoj chemoterapie a dalších (Thathy et al. 1994; Ramirez et al. 1995; Lara Capurro et al. 2000).

Ve své dizertační práci se soustřeďuji na Haemosporida volně žijících ptáků (pro komplexnější pohled z několika různých řádů) a na způsob jejich přenosu hmyzími vektory v přírodních populacích, což byla prozatím opomíjená oblast.

Naše výsledky byly získány jak tradičními metodami (vyšetření infekcí pomocí mikroskopování krevních roztěrů), tak především molekulárně biologickými metodami (detekcí parazitů pomocí nested PCR) založenými na práci s unikátními haplotypy jednotlivých linií haemosporid.

Velkou výhodou také bylo, že jsme ve většině studií sledovali populaci hostitelského druhu dlouhodobě, po několik hnízdních sezón, což umožnilo retrapy stejných jedinců, zjištění jejich infekčního statusu a vytvoření závěrů o chronické fázi malarického onemocnění.

V současné době zažívají molekulárně ekologické studie ptačích haemosporid velký boom, především díky plošnému rozšíření nových metod a jejich relativní cenové dostupnosti. V době vzniku našich prvních publikací se ale jednalo o jedny z úplně prvních publikací věnujících se komplexně vektorům

(převážně tiplíkům jako přenašečům rodu *Haemoproteus* a muchničkám jako přenašečům rodu *Leucocytozoon*) a ptačím maláriím v populaci jednoho druhu ptačího mezihostitele i o jedny z mála studií využívajících kombinovaná mikroskopická a molekulární data k určení prevalencí, druhů a linií ptačích heamosporid.

CÍLE DIZERTAČNÍ PRÁCE

- Určení možných přenašečů haemosporid v České republice a detekce linií haemosporid pomocí molekulárních metod a určení míry jejich hostitelské specificity u těchto přenašečů.

- Získání komplexních informací o prevalenci krevních haemosporid na území České republiky v populacích ptačích mezihostitelů patřících do různých ptačích řádů. A to pomocí převážně molekulárně biologických metod, ale i tradičními mikroskopickými metodami.

- Identifikace genetických linií (unikátních haplotypů) zástupců parazitů způsobujících ptačí malárii vyskytujících se na území České republiky u volně žijících ptáků (na průřezu více ptačích druhů z několika řádů).

POUŽITÉ METODY:

Detekce krevních haemosporid jsme prováděli především molekulárně biologickými metodami.
DNA byla izolována a purifikována z ptačí krve (či jedinců krevsajícího hmyzu odebraných v terénu) pomocí DNeasy® Tissue kitu. Koncentraci a čistotu vyizolované DNA jsme měřili na spektrofotometru NanoDrop® ND-1000 a v případě vysokých koncentrací naředili na koncentraci 5–20 ng/µl.
K detekci parazitů jsme použili dvojstupňovou nested PCR (Hellgren et al. 2004), případně některou z jejích alternativ (Perkins & Schall, 2002; Drovetski et al. 2014), amplifikující 479 bp dlouhý úsek cytochromu *b* (s primery 522 bp). Pro vyhodnocení pozitivity daného vzorku jsme provedli standardní elektroforézu na 2% agarózovém gelu (s použitím negativní kontroly na každých sedm vzorků). Jelikož práce s každým hostitelským druhem i spektrem linií parazitů vyžaduje o něco jiné nastavení podmínek (Cosgrove et al. 2006; Szollosi et al. 2008), byly tyto pro každou ze studií optimalizovány a lehce modifikovány (tak jak je popsáno u jednotlivých původních prací). Pozitivní vzorky byly následně sekvenovány Sangerovým sekvenováním.
Nově identifikované haplotypy (linie), lišící se alespoň jedním nukleotidem od sekvencí uložených v databázích GenBank a MalAvi (Bensch et al. 2009), byly pojmenovány ve shodě s pravidly databáze MalAvi a uloženy do obou zmíněných databází. Ve výzkumných projektech, kde jsme měli k dispozici i krevní roztěry, byla provedena i mikroskopická detekce parazitů. Pro identifikaci parazitů na roztěrech jsem použil světelný mikroskop Axiophot a využil metodu dle Votýpky 2003. K determinaci krevsajících dvoukřídlých jsem použil klíč Chvála 1980.
Ze statistických metod jsme uplatnili především zobecněné lineární modelování (GLM) prováděné v S-PLUS 8.0 (TIBCO Spotfire, Palo Alto, USA), fitován byl vždy plný model se smysluplnými interakcemi. Modely byly vyhodnoceny pomocí likelihood-ratio testů a Chi2 statistiky (Crawley 2007).

PŘEHLED PŮVODNÍCH PRACÍ, ZAHRNUTÝCH DO DIZERTAČNÍ PRÁCE, S POPISEM JEJICH OBSAHU A PŘÍNOSU

I) Synek P, Munclinger P, Albrecht T, Votýpka J (2013a). Avian haemosporidians in haematophagous insects in the Czech Republic. Parasitol Res 112: 839–845. Doi: 10.1007/s00436-012-3204-3

V této publikaci jsme se věnovali krevsajícímu dvoukřídlému hmyzu jako konečným hostitelům a především přenašečům ptačích haemosporid. Podařilo se nám odchytit pomocí CDC pastí se sentinely a následně determinovat 2853 jedinců krevsajících dipter (9 druhů tiplíku rodu *Culicoides*, 5 druhů komárů rodů *Aedes* a *Culex* a jeden druh muchničky – *Eusimulium angustipes*). Následně jsme použili velmi citlivou dvoustupňovou nested PCR a s její pomocí jsme detekovali u těchto vektorů 9 genetických linií (samostatných haplotypů) patřících zástupcům všech třech hlavních rodů ptačích heamosporid. Všechny linie rodu *Leucocytozoon* jsme nalezly pouze v muchničkách a 5 linií rodu *Heamoproteus* jsme zjistili u tiplíků. U komáru jsme zachytili dvě linie, jednu linii rodu *Plasmodium* a překvapivě jednu linii rodu *Haemoproteus*, kdy se velmi pravděpodobně jednalo o aberantní vývoj v nespecifickém vektorovi. Tři linie námi detekovaných haemosporid (dvě linie rodu *Leucocytozoon* a jeden *Haemoproteus*) byly zcela nové a nebyly popsány doposud v jiných studiích. Dvě linie rodu *Haemoprotues*, které jsme detekovali ve vektorech, jsme zachytili také u ptáků na studované lokalitě a to včetně stálých druhů, což naznačuje přenos těmito vektory v místních podmínkách.
V době svého vzniku se jednalo o jednu z prvních studií zabývající se detekcí ptačích heamosporid molekulárními metodami, a to především u tiplíků rodu *Culicoides* jako přenašečů parazitů *Haemoproteus* a muchniček (Simulidae) jako vektorů rodu *Leucocytozoon*. V prostoru střední Evropy se jednalo o vůbec první studii haemosporid u jejich vektorů.

II) Synek P, Albrecht T, Vinkler M, Schnitzer J, Votýpka J, Munclinger P (2013b). Haemosporidian parasites of a European passerine wintering in South Asia: diversity, mixed infections and effect on host condition. Parasitol Res 112: 1667–1677. Doi: 10.1007/s00436-013-3323-5

Tato práce představuje dlouholetou studii ptačích heamosporid (8 po sobě jdoucích hnízdních sezón) populace hýla rudého (*Carpodacus erythrinus*) na okraji jeho hnízdního areálu ve Vltavských luzích na Šumavě. Hýl rudý je z parazitologického hlediska velmi zajímavý druh, protože na rozdíl od

většiny evropských tažných ptáků nezimuje v tropické Africe, ale v Asii. Celkem bylo vyšetřeno na přítomnost krevních haemosporid 240 krevních vzorků patřících 199 dospělým jedincům (v jednotlivých letech se vyskytovaly reptrapy). Celková prevalence ptačích haemosporid byla značně vysoká, celkově dosahovala 60 %, vysoký byl rovněž detekovaných linií (celkem 22 unikátních haplotypů: 5 patřících rodu *Haemoproteus*, 10 rodu *Plasmodium* a 7 rodu *Leucocytozoon*). Zjištěná prevalence se mezi jednotlivými lety pohybovala od 36 do 81 % pro rod *Haemoproteus*, 8–22 % pro *Plasmodium*, a 0–14 % pro rod *Leucocytozoon*. Některé z linií haemosporid (SISKIN1, CCF3 a BT2) zjištěných u hýlů jsme detekovali i u stálých ptáků na lokalitě, což naznačuje jejich lokální přenos. Naopak jiné linie (FANTAIL1 a EMSPO05) jsou známy pouze z asijských ptačích hostitelů, což indikuje, že k přenosu pravděpodobně došlo na zimovištích hýla v Asii. Část linií vykazovala striktní hostitelskou specificitu (např. nejčastěji zastoupená linie ROFI2), jiné byly silně generalistické (např. SGS1).

Zjišťovali jsme také vliv parazitace na nejrůznější charakteristiky napadených jedinců. Zjistili jsme, že infekce studovanými krevními parazity nemá vliv na celkovou hmotnost napadených jedinců či na karotenoidní ornament samců hýla rudého.

III) Synek P, Popelková A, Munclinger P, Koubínová D, Šťastný K, Langerová I, Votýpka J (2016). Haemosporidian infections in the Tengmalm's Owl (*Aegolius funereus*) and potential insect vectors of their transmission. Parasitol Res 115: 291–298. Doi: 10.1007/s00436-015-4745-z

V této publikaci jsme použili molekulární metody ke studiu diverzity ptačích haemosporid hnízdní populace sýce rousného v Krušných horách. Celkem jsme identifikovali 5 linií haemosporid, jedna z nich byla zcela nová. Protože jsme opakovaně nacházeli parazity i u mláďat na hnízdech v budkách (nejmladší infikované mládě bylo 12 dní staré), instalovali jsme do oddělené části budek lepové pasti k monitoringu a zachycení krevsajících dipter. Tyto potenciální přenašeče jsme vyšetřili na přítomnost ptačích haemosporid a detekovali u nich 6 linií těchto parazitů (jedna linie byla nová). Jedna z těchto linií se vyskytovala jak u mláďat sýců, tak v odchyceném hmyzu a indikovala tedy lokální přenos na lokalitě pomocí dvou druhů muchniček (*Nevermannia vernum* a *Eusimulium angustipes*).

Spoluautorské publikace:

IV) Svobodová J, Gabrielová B, Synek P, Maršík P, Vaněk T, Albrecht T, Vinkler M (2013). The health signalling of ornamental traits in the Grey Partridge (*Perdix perdix*). J Ornithol 154: 717–725. Doi: 10.1007/s10336-013-0936-5

Tato studie se zabývala především karotenoidním ornamentem na kůži koroptví polních (*Perdix perdix*) a melanoidním ornamentem na jejich peří jako možnými prediktory jejich individuální zdatnosti, zdravotní kondice a funkce imunitního systému. Statut infekce ptačími haemosporidy měl představovat jednu ze sledovaných charakteristik. Nakonec ale byla prevalence ptačí malárie u sledovaného vzorku velmi nízká (12 % z 50 jedinců), což neumožnilo pokročilejší statistické vyhodnocení. Byla ale charakterizována parazitofauna koroptví molekulárními metodami a zjištěna překvapivě nízká diverzita krevních parazitů (všichni nakažení jedinci byly infikovaní jedinou linií SGS1 patřící k morfologickému druhu *Plasmodium relictum*).

V) Hanel J, Doležalová J, Stehlíková Š, Modrý D, Chudoba J, Synek P, Votýpka J (2016). Blood parasites in northern goshawk (*Accipiter gentilis*) with an emphasis to *Leucocytozoon toddi*. Parasitol Res 115: 263–270. Doi: 10.1007/s00436-015-4743-1

Tato práce byla první komplexní studií popisující haemosporida u populace jestřába lesního (*Accipiter gentilis*) pomocí molekulárních metod. Populace ptačího mezihostitele byla vzorkovaná po dobu pěti let a byly odebrány vzorky jak z dospělců, tak mláďat na hnízdech. Opět kombinovala jak molekulární detekci parazitů pomocí nested PCR, tak mikroskopování krevních roztěrů. Dospělí ptáci vykazovali velmi vysoké prevalence, mláďata pak výrazně nižší. Celkem jsme detekovali pět unikátních genetických linií haemosporid. Tři z linií identifikované u jestřábů v této studii (a dokonce ty nejčastěji zastoupené) byly zcela nové a prozatím se zdají být pro jestřáby unikátní, další dvě, podstatně méně zastoupené linie, patří ke generalistickým parazitům známým z mnoha dalších ptačích druhů.

SOUHRNNÉ VÝSLEDKY PRACÍ ZAHRNUTÝCH DO DIZERTAČNÍ PRÁCE V KONTEXTU SOUČASNÉHO POZNÁNÍ

Jednotlivé výsledky jsou detailně popsány v publikacích, které jsou součástí této dizertační práce (viz níže). Zde se zaměřuji na shrnutí výsledků, které byly jednotícím tématem předkládaných prací.

Na území České republiky byly prozatím krevní haemosporida studovány spíše sporadicky a převážně jen pomocí tradičních mikroskopických metod (Kučera 1981a, b; Svobodová & Votýpka 1998; Závodská et al. 2004; Svobodová et al. 2015). Poslední tři zmíněné studie se věnovali pouze haemosporidům u dravců. Molekulární detekci linií haemosporid u nás prováděl Svoboda et al. 2015, který u 95 jedinců slavíků modráčků (*Luscinia svesica*) identifikoval 6 linií rodu *Plasmodium* (DEURB05, GRW04, GRW06, GRW11, SGS1 a TURDUS1), 3 linie rodu *Leucocytozoon* (BT1, BT2 a BT6) a jednu linii rodu *Haemoproteus* (WW2). České vzorky byly také součástí datasetu Marzala et al. 2011, kde u 50 jedinců vrabce domácího (*Passer domesticus*) nalezl linie plasmodií SGS1 a GRW11, a vzorky českých chřástálů polích (*Crex crex*) byly součástí studie Fourcade et al. 2014, který u 24 jedinců detekoval 2 linie rodu *Plasmodium* (GRW06 a SYBOR10) a jednu rodu *Leucocytozoon* (RSTR1).

My jsme v rámci našich studií (Synek et al. 2013b; Svobodová et al. 2013; Synek et al. 2016; Hanel et al. 2016), které jsou součástí předkládané dizertační práce, vyšetřili na přítomnost krevních haemosporid 522 jedinců ptačích mezihostitelů ze čtyř řádů (pěvci – Passeriformes, sovy – Strigiformes, dravci – Accipitriformes a hrabaví – Galliformes). U ptačích hostitelů jsme detekovali celkem 27 linií (unikátních haplotypů) všech tří hlavních rodů ptačích haemosporid (*Plasmodium*, *Haemoproteus* a *Leucocytozoon*), z nichž 8 bylo zcela nových.

Z linií zachycených v předchozích studiích na území České republiky jsme u dvou ptačích druhů (hýl rudý a koroptev polní) detekovali rovněž linii plasmodia SGS1 patřící morfologickému druhu *Plasmodium relictum* (Palinauskas et al. 2007). A to i přesto, že jsme studovali jiné hostitelské druhy než předchozí zmíněné studie (Marzal et al. 2011, studující vrabce domácí a Svoboda al el. 2015, studující slavíky modráčky). Tento fakt je možné vysvětlit velmi nízkou hostitelskou specificitou linie SGS1 a její schopností infikovat hostitele dokonce napříč různými ptačími řády (Robalinho Lima & Bensch 2014). Podobnými generalisty jsou i linie rodu *Plasmodium* TURDUS1, reprezentující morfologický druh *P. circumflexum* (Palinauskas et al. 2007), a linie GRW06 ztotožněná s druhem *P. elongatum* (Valkiūnas et al 2008). Linii TURDUS1 jsme detekovali u jestřábů a sýců, z našeho

území je známá rovněž ze slavíků modráčků (Svoboda et al. 2015), jedná se o silně generalistickou linii napadající ptáky ze 13 čeledí čtyř řádů (např.: Paulinkas et al. 2013; Huang et al. 2015; Dubiec et al. 2016; Ciloglu et al. 2016). Linie GRW06 námi detekovaná u hýlů rudých a autory předchozích studií z území ČR také u slavíků (Svoboda et al. 2015) a chřástalů (Fourcade et al. 2014) infikuje dokonce ptáky ve 32 čeledích z 12 řádů a svým výskytem je značně kosmopolitní (např.: Okanga et al 2014; Illera et al 2015; Niebuhr et al 2016; Ramey et al 2016; Seimon et al 2016; Fecchio et al 2017). Mezi leucocytozoony je podobným generalistou linie BT2, která byla detekována na českém území u slavíků modráčků (Svoboda et al. 2015) a v našich studií se vyskytovala u sýců, hýlů i jestřábů a je známá i z mnoha jiných lokalit napříč celou Evropou (Santiago-Alargon et al. 2011; Hellgren et al. 2013; Van Rooyen et al. 2013; Rojo et al. 2014)

Vyšetřených krevních vzorků bylo v našich studiích celkově ještě více (597), díky tomu, že většina našich studií byla dlouhodobých: konkrétně studie hýla rudého osmiletá (Synek et al. 2013b), jestřábů pětiletá (Hanel et al. 2016) a sýců čtyřletá (Synek et al. 2016) a docházelo k retrapům, tj. opakovanému odchycení stejných individuí během několika sezón. To nám umožnilo studovat jejich infekční statut po dobu několika let a pozorovat jeho eventuální změny. Většinou zůstal infekční statut neměnný, méně často docházelo k získání nové linie parazita (či více linií) a nejméně často ke ztrátě infekčního statutu pro danou linii v následujících letech. Tyto výsledky by podporovali hypotézu, že v případě úspěšného přenosu je parazit přítomen v hostiteli po celou dobu jeho života (Valkiūnas 2005). Haemosporida se vyskytují v periferní krvi v nízkých počtech, ale při oslabení jedince stresem či nákaze jinou infekcí se může dočasně tento počet výrazně zvýšit a paraziti jsou tedy snáze detekovatelní (Remple 2004).
Vzácnější byly případy ztráty infekčního statutu jedince určovaného z periferní krve. Tento stav (ztráta pozitivního statutu) popisuje i v několika málo dalších pracích studující ptačí populace více po sobě jdoucích let (Bensch et al. 2007, Knowles et al. 2011). Nejčastěji to bývá vysvětlováno životním cyklem těchto parazitů: vyskytují se zde stádia, která jsou skryta ve vnitřních orgánech mezihostitele (Valkiūnas 2005; Zehtindjiev et al. 2008) a absence gametocytů v krvi tudíž nemusí znamenat kompletní uzdravení jedince, ale jen přítomnost parazita v merogoniální fázi cyklu v jiných orgánech než periferní krvi (Mendes et al. 2013).

Náš hlavní zájem směřoval k vektorům ptačích haemosporid. Vyšetřili jsme celkem 3054 jedinců krevsajících dipter jako potencionálních přenašečů a konečných hostitelů těchto parazitů. U vektorů jsme detekovali 12 unikátních linií ptačích haemosporid a z nichž čtyři byly nové.
Celkem jsme vyšetřili vzorky z 2620 jedinců tiplíků rodu *Culicoides*, u těchto vektorů se jednalo o jednu z prvních studií studující jejich haemosporida molekulárními metodami. Tiplíci jsou obecně

považováni za přenašeče parazitů rodu *Haemorpoteus* (Valkiūnas 2005), velmi vzácně i rodu *Leucocytozoon* (Morii 1992). Druhy parazitů rodu *Haemoproteus*, jež byly ve vztahu k jejich vektorům nejvíce studovány (*H. mansoni*, *H. danilewskyi*), využívají za přenašeče větší počet druhů tiplíků r. *Culicoides* (Atkinson 1988, 1991; Garvin a Greiner 2003). Valkiūnas et al. (2002) zase ukázal, že jeden druh tiplíka (konkrétně *C. impunctatus*) může sloužit jako vektor až pro pět druhů parazitů rodu *Haemoproteus*. Tyto výsledky pak byly potvrzeny i pro další druhy (Valkiūnas & Iezhova 2004).Všechny předchozí studie ale využívaly tradiční mikroskopické metody a experimentální nákazy v laboratoři. Pomocí molekulárních metod na úrovni genetických linií přenášených haemoproteů tiplíky jako první studoval Martinez-de la Puente et al. 2011, a naše studie (Synek at al. 2013b), která je součástí předkládané dizertační práce, byla druhou studií v celosvětovém měřítku.

V našich studiích jsme zjistili, že tiplík druhu *Culicoides segnis* může být přenašečem až čtyř linií rodu *Haemoproteus* a tiplík *C. kibunensis* tří linií. A rovněž identické linie haemoproteů jsme detekovali až ve třech druzích tiplíků, čímž jsme ukázali, že hostitelská specificita linií nemusí být omezena pouze na jeden konkrétní druh tiplíka. Tyto závěry odpovídaly i tomu, co v předchozí studii zjistil Martinez-de la Puente et al. 2011. Na tyto dvě studie (Martinez-de la Puente et al. 2011, Synek et al. 2013a) v rychlém sledu navázali další studie, které potvrdili naše domněnky (Bobeva et al. 2013; Ferraguti et al. 2013a; Bobeva et al. 2014; Bukauskaitė et al 2015). Závěry získané těmito studiemi z terénu pak v experimentálních podmínkách v laboratoři při kontrolovaných přenosových experimentech potvrdil Žiegytė et al. 2017.

Komáři (Culicidae) jsou přenašeči krevních haemosporid rodu *Plasmodium* (Valkiūnas 2005). Ptačí plasmodia se v rámci svých vektorů obecně vyznačují nízkou hostitelskou specificitou, což bylo prokázáno u mnoha studií z různých částí světa (Work et al. 1990; La Pointe et al. 2005; Ejiri et al. 2009; Kimura et al. 2010; Ishtiaq et al. 2008; Ferraguti et al. 2013b; Schoener et al. 2015). Jeden druh komára je rovněž schopen hostit více linií plasmodií (Kimura et al. 2010; Glaizot et al. 2012; Zittra et al. 2015) a obecně u komárů dochází často k výskytu aberantního vývoje, kdy se ve vektorech po určitou dobu vyvíjí parazit, který svůj vývoj ale nedokončí a není schopen infikovat ptačího mezihostitele (Valkiūnas 2011; Žiegytė & Valkiūnas 2014). Tuto skutečnost jsme zaznamenali i v naší studii (Synek et al. 2013a), kdy jsme u komárů *Culex pipiens* detekovali i jednu z linií rodu *Haemoproteus*. Domníváme se, že se velmi pravděpodobně jednalo o aberantní přenos. V naší práci jsme navrhli experimentální ověření této skutečnosti, a to v kontrolovaných laboratorních přenosech s pitvami, což bylo v následujících letech několikrát provedeno a aberantní vývoj parazitů rodu *Haemoproteus* byl v komárech opakovaně potvrzen (Valkiūnas et al. 2013, 2014).

U muchniček (Simuliidae) jako přenašečů rodu *Leucocytozoon* (Valkiūnas 2005) existuje velmi málo studií, které pracovaly s nenasátými samicemi. Naše studie (Synek et al. 2013a) byla tak jednou

z prvních v této oblasti. Identifikovali jsme tři linie rodu *Leucocytozoon* v jednom druhu muchničky a ve druhé studii (Synek et al. 2016) čtyři linie u druhu *Nevermannia vernum*, přitom linie NEVE1 se nacházela i u muchniček rodu *Eusimulium*. Před námi studoval na úrovni genetických linií molekulárními metodami nenasáté muchničky jako přenašeče rodu *Leucocytozoon* pouze Sato et al. (2009), který zjistil identickou linii rodu *Leucocytozoon* u tří druhů muchniček dvou rodů. Další studie na toto téma se objevily až po naší studii, ale všechny potvrzují, stejně jako naše data, schopnost jednoho druhu muchniček sloužit jako vektor mnoha linií rodu *Leucocytozoon* (Murdock et al. 2015; Woodford et al. 2018). Tyto výsledky korespondují i s nálezy ze studie Desser & Bennet (1993), kteří experimentálně zjistili, že jeden druh muchničky může přenášet až 5 různých druhů rodu *Leucocytozoon*.

ZÁVĚR

Jako potencionální přenašeče krevních haemosporid na území České republiky jsme určili tiplíky druhů *Culicoides kibunensis*, *C. segnis* a *C. festivipennis*, komáry komplexu *Culex pipiens* a muchničky druhů *Eusimulium securiforme*, *E. angustipes* a *Nevermannia vernum*. Nalezli jsme u nich celkem 12 linií ptačích haemosporid, z nichž čtyři linie byly nové. Naše data získaná na tiplících a muchničkách byla jedněmi z prvních získaných molekulárními metodami a popisujícími unikátní genetické linie haemosporid v hmyzích vektorech. Na tyto naše práce s rozvojem zavádění molekulárně biologických metod do studia přenašečů haemosporid následně navázalo velké množství dalších studií. U ptačích mezihostitelů jsme popsali krevní haemosporida u populací čtyř druhů ptáků ze čtyř různých řádů (pěvci – Passeriformes, sovy – Strigiformes, dravci – Accipitriformes a hrabaví – Galliformes). Prevalence se pohybovaly od 12 % u koroptví polních (*Perdix perdix*) po 86 % v populaci sýce rousného (*Aegolius funereus*). U ptačích hostitelů jsme detekovali celkem 27 linií (unikátních haplotypů) všech tří hlavních rodů ptačích haemosporid (*Plasmodium*, *Haemoproteus* a *Leucocytozoon*), z nichž 8 bylo zcela nových.

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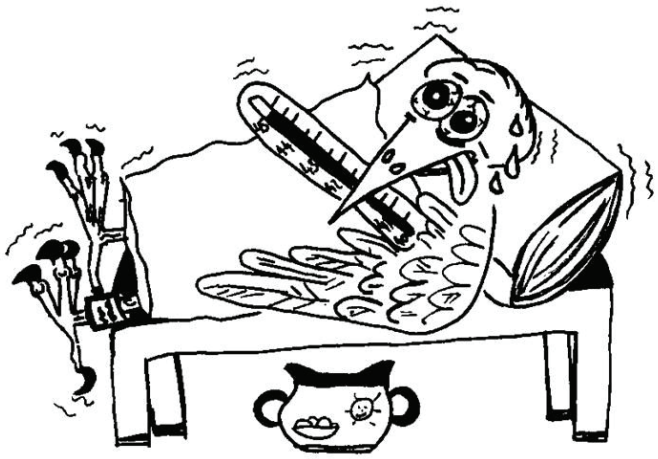

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ORIGINAL PAPER

Avian haemosporidians in haematophagous insects in the Czech Republic

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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.

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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.

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

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ORIGINAL PAPER

Haemosporidian parasites of a European passerine wintering in South Asia: diversity, mixed infections and effect on host condition

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Abstract We studied haemosporidian parasites in the scarlet rosefinch *Carpodacus erythrinus* in a small isolated semicolony during an eight-year period using molecular methods of parasite detection. The scarlet rosefinch is an interesting model of parasite host species. It winters in South Asia which represents a rare exception among European passerines. Males express yellow to red carotenoid-based plumage ornament which is a good predictor of male reproductive success. In 240 blood samples originating from 199 adult individuals, the total parasite prevalence reached 60 %. Prevalence varied among years from 36 to 81 % in *Haemoproteus*, 8 to 22 % in *Plasmodium*, and 0 to 14 % in *Leucocytozoon*. Twenty parasite lineages were detected (*Haemoproteus*: 5 lineages, *Plasmodium*: 10 lineages, and *Leucocytozoon*: 5 lineages). Among them, the *Haemoproteus* ROFI2 lineage, which is a host-specific parasite lineage of the scarlet rosefinch, was the most frequently found. Parasite lineages showed varying degree of lineage specificity. While *Haemoproteus* lineages detected in the scarlet rosefinch have relatively narrow host breadth restricted mainly to Fringillidae family, *Leucocytozoon* and *Plasmodium* lineages generally showed wider host range. The presence of some parasite lineages hitherto detected in sedentary European passerines (SISKIN1, CCF3, BT2) or in *Culicoides* biting midges at the same locality (ROFI1) suggest local transmission. On the contrary, lineages LK05 and FANTAIL1 that were previously reported exclusively from Asian hosts imply parasite transmission at the scarlet rosefinch wintering sites in South Asia. Mixed infections were found in 17 % of infected samples and comprised mainly the most frequent lineages. The pattern of concomitant infections seemed to be rather random and matched expected levels based on lineage frequencies. Between-year comparisons revealed that in a majority of the repeatedly captured individual hosts the infection status remained unchanged (individuals stayed uninfected or possessed the same parasite lineages). However, 16 gains and 8 losses of lineages were also reported. We have not found any effect of haemosporidians on male carotenoid ornament expression or host body mass.

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Introduction

Haemosporidians, frequent blood parasites of birds transmitted by blood-sucking Dipterian vectors, are diverse group comprising more than 200 described species classified into several genera: *Plasmodium*, *Haemoproteus* and *Leucocytozoon*, which can cause bird disorders (Bennett et al. 1993). Molecular methods of parasite detection (Bensch et al. 2000; Waldenström et al. 2002; Richard et al. 2002; Fallon et al. 2003; Hellgren et al. 2004) started exponential grow of published studies covering various aspects of

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haemosporidian diversity, biology, host specificity, and effect on host fitness. Despite this extensive effort, our current view on many aspects of the host–parasite relationships (including parasite lineage specificity and the effect of parasites on host condition) has remained unclear.

Haemosporidian cytochrome *b* lineages which were shown to represent distinct evolutionary entities (Bensch et al. 2004, Valkiūnas et al. 2008a, Valkiūnas et al. 2008b) differ considerably in avian-host specificity. While *Plasmodium* lineages often develop in a large range of phylogenetically unrelated hosts (Waldenström et al. 2002; Križanauskienė et al. 2006; Hellgren et al. 2007; Beadell et al. 2009) and are able to spread between continents via frequent shifts from resident to migratory hosts (Hellgren et al. 2007), *Haemoproteus* lineages tend to show narrower host specificity (Bensch et al. 2009). Moreover, large variability in host specificity exists even among members of each genus. *Plasmodium* lineage SGS1 (assigned to *Plasmodium relictum* morphospecies) infecting almost 50 host species classified into several bird orders and some *Haemoproteus* or *Plasmodium* lineages found in single host species (Bensch et al. 2009) can serve as examples of the extreme limits in parasite–host specialization variability. Typically, Haemosporidian lineage spectra found in a particular host species consist of only few abundant parasite lineages and an array of infrequent lineages. These rare lineages may sometimes represent accidental transmissions into new hosts that cannot result in full development of the parasite and thus form a dead end in the parasite cycle (Valkiūnas et al. 2009a, Valkiūnas 2011). Since the rare infections are frequently transmitted to migratory birds at their wintering grounds where the regular local resident hosts may be known, lineage identity can provide interesting insights into the migratory host biology, especially in species in which the migration pathways are poorly documented.

Experiments performed under controlled laboratory conditions using mainly *Plasmodium* lineages repeatedly showed important pathogenic impact of haemosporidians on their avian hosts (Valkiūnas et al. 2006; Zehtindjiev et al. 2008; Palinauskas et al. 2011). However, the situation in nature seems to be more complex. While some studies demonstrated a significant effect of these haematobious parasites on bird fitness and/or condition (e.g. Seutin 1994; Merila and Sheldon 1999; Merino et al. 2000, Hõrak et al. 2001; Marzal et al. 2005, Westerdahl et al. 2005; Kilpatrick et al. 2006, Knowles et al. 2010), other studies failed to find any effect of this kind (e.g. Dale et al. 1996; Gonzalez et al. 1999; Votýpka et al. 2003; Deviche et al. 2005; Gibb et al. 2005; Bensch et al. 2007). This incongruity probably stems from the following reasons: (1) wild birds usually exhibit low levels of parasitemia suggesting chronic phase of infection which can only slightly affect the host (Valkiūnas 2005, Asghar et al. 2011), (2) while the parasite transmission, which is followed by acute phase of disease, frequently occurs at wintering grounds, host fitness or condition are measured mostly during the breeding season (Bensch et al. 2007), (3) parasites may be absent in peripheral blood in certain stages of their development and, thus, their detection using blood samples may be problematic (Zehtindjiev et al. 2008), (4) mixed infections which complicate the analyses are frequent (Marzal et al. 2008), and (5) studies may be biased by lower probability of sampling infected then uninfected birds (Valkiūnas 2005).

In our study, we have focused on investigation of haemosporidian parasites in a small community of scarlet rosefinch (*Carpodacus erythrinus*) breeding in the Czech Republic. In eight successive years, we trapped and sampled nearly all individuals at the breeding ground. Scarlet rosefinch is a small, semi-colonial, sexually dimorphic cardueline finch with delayed plumage maturation in males (Stjernberg 1979). Species breeding range spans from central Europe to Kamchatka and covers large area of the Palaearctic temperate zone. In the Czech Republic, the scarlet rosefinch breeds at the westernmost limit of its distribution which has been established by recent expansion of the species breeding range (Cramp et al. 1994). The scarlet rosefinch represents an interesting model of a haemosporidian host species for the following two reasons: (1) it is sexually dichromatic, with yellow to red carotenoid-based plumage of males signaling condition and male reproductive success (Albrecht et al. 2007, Vinkler et al. 2012). Hence, the male quality can be easily assessed, and the influence of parasites on male sexual traits can be studied. (2) Avian malaria research is strongly biased towards European–African and American migratory system. Scarlet rosefinch wintering grounds are located in South Asia which represents a rare exception among passerines breeding in Europe. Among birds breeding in Europe and wintering in South Asia, the paddyfield warbler *Acrocephalus agricola* has been the only species studied extensively for avian malaria using molecular methods (Zehtindjiev et al. 2009). Haemosporidian parasites in the scarlet rosefinch have been so far studied only marginally within the framework of comparative studies dealing with limited number of rosefinch individuals (Beadell et al. 2006, Križanauskienė et al 2006; Hellgren et al. 2007, Pérez-Tris et al. 2007). Four haemosporidian lineages have been hitherto detected in the scarlet rosefinch: *Haemoproteus* ROFI1 and ROFI2, and *Plasmodium* BT7 a SGS1. In our study population, we previously described the impact of *Haemoproteus* infection (detected by microscopy) on individual haematological state (Vinkler et al. 2010). In the present study, we used molecular methods to detect and precisely identify haemosporidian lineages in the same breeding population, estimate parasite prevalence and its temporal variation, reveal mixed infections, and examine the impact of infections on host body mass and male ornamentation.

Material and methods

Fieldwork

As the locality and field procedures were described in detail elsewhere (Albrecht 2004; Albrecht et al. 2007 and 2009; Vinkler et al. 2012), we mention here both only briefly. Rosefinches were studied from May to July during the years 2001–2008 at an isolated patch of wet shrubby meadow situated in the Vltava river valley (48°49' N; 13°56' E) in the Bohemia Forest National Park in Doudlebia, Czech Republic. Since the vegetation suitable for rosefinch nesting was searched systematically several times at the locality every year during the egg-laying period, almost all nests have been detected. Adults were mist-netted upon arrival to the locality or during the nest provisioning phase. Caught birds were transported into a field laboratory where basic measurements were taken (weight, tarsus length) and small amount of blood was obtained via brachial venipuncture and stored in ethanol. Breast patch colour (as the most important male ornamentation trait in this species) of males in their third year or older was measured using digital photography and subsequent computer analyses (all described in Albrecht et al. 2009). Shortly, photographs were taken under standard conditions and colour and grey charts were used to further standardize the measurements of colours. Hue, saturation and lightness of carotenoid ornament were measured (HSB colour space) using ADOBE PHOTOSHOP software (Adobe Systems Inc., San Jose, California). We also collected 17 individual blood samples of 13 other bird species trapped accidentally at the study plot (Appendix 1).

Parasite detection

All blood samples were dried in the laboratory and DNA was extracted using DNeasy® Tissue Kit (Qiagen). Presence and quality of the extracted host (Rosefinch) DNA was inspected by spectrophotometer NanoDrop® ND-1000 (Isogen Life Science) and by control PCRs using mitochondrial DNA (control region and ND2) primers (Pavlova et al. 2005) followed by agarose gel electrophoresis. Parasites were detected following the nested PCR protocol described in Hellgren et al. 2004 (see also Synek et al. 2013 for details), which enables to distinguish *Plasmodium* or *Haemoproteus* infections from *Leucocytozoon* ones using genera-specific nested primers. Positive samples were sequenced using primers HaemF (designed for *Plasmodium* or *Haemoproteus*) or HaemFL (designed for *Leucocytozoon*) (Hellgren et al. 2004). Parasite lineages were identified and classified according to MalAvi database (Bensch et al. 2009). All new haplotypes differing by one or more substitutions from the sequences deposited in GenBank were sequenced also from the 3′-end with primers HaemR2 and HaemR2L designed for *Plasmodium* or *Haemoproteus* and *Leucocytozoon* respectively. Since the two previously described haemosporidian lineages found in the scarlet rosefinch were named ROFI1 and ROFI2 (using contraction of the word rosefinch), we followed this habit and started the names of the new lineages with ROFI followed by numbers from 3 to 7 as recommended by Pérez-Tris et al. (2007). Sequences of the new lineages were deposited in GenBank (Accession numbers from JX556907 to JX556911).

The basic protocol used (Hellgren et al. 2004) enables simple detection of mixed infection of parasite lineages belonging to different genera (infection by a *Leucocytozoon* lineage and concurrently by *Plasmodium* or *Haemoproteus* lineage). However, mixed infections of *Haemoproteus* and *Plasmodium* lineages or mixed infections of single genus lineages require additional treatment (Loiseau et al. 2008; Marzal et al. 2008). Two strategies were adopted to distinguish these infections: (1) we checked carefully chromatograms for double peaks and (2) we designed lineage-specific primers (Table 1). PCR product of the initial PCR from standard protocol (Hellgren et al. 2004) was used as a template for nested amplifications with lineage-specific primers. Thermal conditions were the same as for the nested PCR in the standard protocol (Hellgren et al. 2004). In each examined sample, presence of the PCR product was verified using an agarose gel. The lineage-specific-primers strategy allowed us to identify mixed infections of *Plasmodium* and *Haemoproteus* lineages and also to detect mixed infections of the most common *Haemoproteus* lineage ROFI2 with other *Haemoproteus* lineages.\

Statistic analysis and other computations

We used generalized linear models (GLM) with identity link function (assuming normal distribution of dependent variables) for statistical evaluation of host–parasite interactions. Full models with reasonable two-way interactions involving the sex of the birds (where both sexes were treated) were fitted. Simplification of full models started with removing interactions and then the main effect (if they were not part of significant interaction). Hence, model terms were removed gradually, one by one, and models with and without the term of interest were compared using likelihood-ratio tests and F statistics (Crawley 2007). Presented are minimal adequate models (MAM) that means model with all members significant ($\alpha<0.05$). Each individual bird was used only once for the analysis (for those who were captured repeatedly, we used the infection status and other data from the first year when an individual was investigated). Analyses were made in R 2.14.2 software (Mathsoft 2011).

Table 1 Lineage-specific primers

Primer name	Sequence (from 5′ to 3′)	Product size (bp)	Parasite lineage(s) amplified
SelPlasF	CATGCAACWGGTGCWTCATT	271	*Plasmodium* lineages
SelPlasR	TTTTTAAGGTTGGGTCACTTACAAG		
SelHaemF	ATTGTTACYGCTTTYATGGGTTA	150	*Haemoproteus* lineages
SelHaemR	TCTTTTTAAAGTTGGATCACTWATAGT		
SelH62F	ATATGCATGCTACTGGAGCTA	477	*Haemoproteus* ROFI2 Lineage
SelH62R	AATAAACTTTGTGCTAAAAATATA		
SelH392F	TGCTACCGGTGCTACATTTG	471	*Haemoproteus* lineages CCF3, SISKIN1, ROFI1, ROFI3
SelH392R	AATAAACTTTGTGCTAGAAATAGG		

Results

Two hundred forty blood samples originating from 199 adult scarlet rosefinch individuals (some birds were captured repeatedly in different years) were analysed. We detected at least one haemosporidian lineage in 145 (60 %) samples. The total number of infections was even higher due to mixed infections (Table 2). Sequencing revealed 20 parasite lineages (Table 2) belonging to three haemosporidian genera: *Haemoproteus* (5 lineages), *Plasmodium* (10 lineages) and *Leucocytozoon* (5 lineages). Five of these lineages were newly identified and these differed by one to eight substitutions from the lineages described previously (Table 3). Individual lineages varied considerably in their prevalence: from dominant ROFI2 which was found in almost half of the samples analysed (112 positive samples, 47 %) to eight rare lineages in which each was detected in a single sample. Apart from ROFI2, only two lineages (SGS1 and BT2) exceeded the level of 5 % prevalence (both lineages 5.4 %). Hence, the pattern of

Table 2 Haemosporidian lineages found in the scarlet rosefinch. Number of infected individuals is given for particular lineage and year

Lineage/year (no. of samples)	2001 (28)	2002 (24)	2003 (21)	2004 (22)	2005 (37)	2006 (34)	2007 (37)	2008 (37)	Total (240)
Haemoproteus									
ROFI2	10	11	15	15	14	13	22	12	112
CCF3		1	1	1	1				4
SISKIN1				1					1
ROFI1			1					2	3
ROFI3					1		1		2
Plasmodium									
SGS1	2	1	1	2	1	2	1	3	13
PADOM02					2	1	2		5
WW3	1	1			1	1		1	5
ROFI4		1							1
TURDUS1								1	1
ROFI5								1	1
BT6								2	2
FANTAIL01		1	1						2
LK05						1			1
BT8					1				1
Leucocytozoon									
BT2	2	1	1	1		3	4	1	13
BT5		1							1
EMSPO05						1			1
ROFI6				1				1	2
ROFI7							1	1	2
Total	15	18	20	21	21	22	31	25	173

Table 3 New lineages

New lineage	Closest formerly described lineage	Closest lineage host(s)	Difference between lineages
Haemoproteus ROFI3	DENPEN02	*Dendroica pensylvanica*[a]	3 substitutions (0.6 %)
Plasmodium ROFI4	MOALB02	*Motacilla alba*[b]	1 substitution (0.2 %)
		Acrocephalus orientalis[b]	
Plasmodium ROFI5	MYITYR01	*Myiarchus tyrannulus*[b]	6 substitution (1.2 %)
Leucocytozoon ROFI6	EMSPO04	*Emberiza spodocephala*[c]	3 substitutions (0.6 %)
	EMSPO05		
Leucocytozoon ROFI7	SILUT01	*Sicalis luteola*[d]	8 substitutions (1.7 %)

[a] Ricklefs and Fallon 2002; Outlaw and Ricklefs 2009

[b] Beadell et al. 2006

[c] Palinauskas et al. unpublished

[d] Merino et al. 2008

lineage prevalence is markedly right-skewed. Parasite prevalence varied among years from 36 to 81 % in *Haemoproteus*, 8 to 22 % in *Plasmodium*, and 0 to 14 % in *Leucocytozoon* (Fig. 1).

While single-parasite lineage was found in 120 blood samples (83 % of positive samples), mixed infections were detected in 25 samples (17 %). Two lineages were found in most samples showing mixed infections (22 samples); however, three different lineages (one *Haemoproteus*, one *Plasmodium*, and one *Leucocytozoon* lineage) were found in three samples corresponding to three different birds (two females and one male). Mixed infection usually comprised the most frequent lineages (ROFI2 96 % of mixed infections, SGS1 24 %, BT2 24 %). Co-occurrence of lineages in mixed infections matched closely expected values calculated from lineage prevalence (Table 4). Expected total number of concomitant infections (cases when particular lineage shares its host with other lineage) did not differ significantly from observed value (expected 29, observed 28).

Thirty birds were trapped repeatedly (41 retraps) in consecutive years. The interval between repeated trappings of a particular individual spanned from 1 to 3 years. Four birds which occurred at locality in two different years remained uninfected. Three of them were re-trapped after two years (which suggests they bred one year outside our study plot). It means they were exposed to potential transmission for at least three breeding and wintering seasons. Apart from these uninfected birds, between-year comparisons revealed that in 20 infected individuals the infection status remained unchanged (they harboured the same lineages). However, we also detected 16 gains and 8 losses of lineages in host bloodstream. Losses involved lineages of all three genera (five lineage losses of *Haemoproteus*; one *Plasmodium*, two *Leucocytozoon*). Losses and gains also comprised lineage replacements: one ROFI2 loss was accompanied by gain of BT2, once FANTAIL01 was replaced by ROFI2, and one individual possessing originally ROFI2, BT2, and SGS1 was negative for ROFI2, BT2 and positive for SGS1 and ROFI1 in the next year.

Close inspection of the scarlet rosefinch lineages in the MalAvi database (Bensch et al. 2009) showed varying degree of lineage specificity (Appendix 2). Apart from five

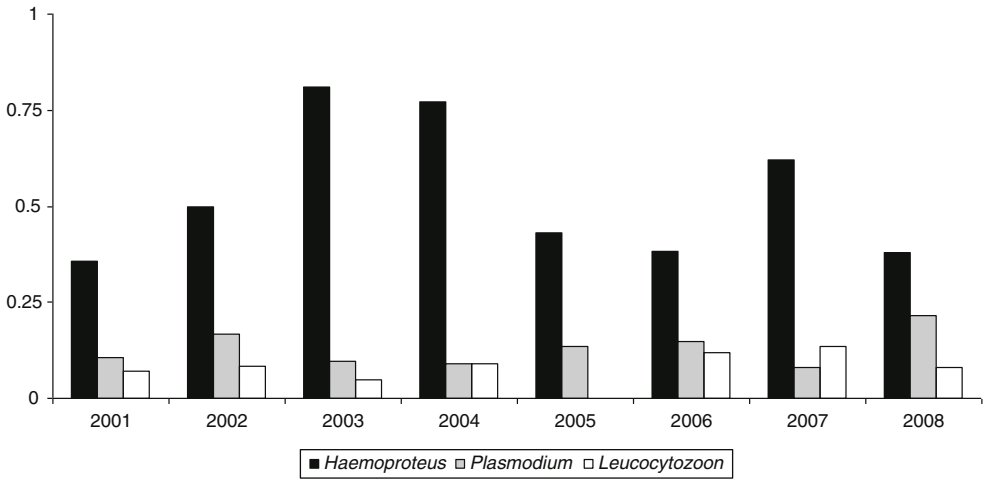

Fig. 1 Temporal changes of parasite prevalence

Table 4 Co-occurrence of parasite lineages. Number of mixed infections is given for particular lineage combination. Expected numbers based on lineage prevalence are given in brackets

	BT2	BT5	ROFI6	ROFI7	SGS1	PADOM02	WW3	ROFI4	TURDUS1	BT6	LK5	CCF3	ROFI3
ROFI2	6[a](6)	1[b](0)	2(0)	2[c](1)	5[a](6)	1(2)	1(2)	1[b](0)	1[c](0)	2(1)	1(0)	2(2)	2(1)
ROFI1					1(0)								

[a] Found also in mixed infection of ROFI2, BT2, and SGS1

[b] Found also in mixed infection of ROFI2, BT5, and ROFI4

[c] Found also in mixed infection of ROFI2, ROFI7, and TURDUS1

infrequent novel ROFI lineages, the most frequent lineage ROFI2 has not been reported in any other avian host species. While *Haemoproteus* lineages detected in our study were found exclusively in European passerines of the Fringillidae family (with the exception of SISKIN1 which was found in the house finch *Carpodacus mexicanus* and also the house sparrow *Passer domesticus* in the North America), *Plasmodium* and *Leucocytozoon* lineages show lower host specificity, including even non-passerine birds. They also often exhibit larger geographic distribution. However, *Plasmodium* lineage FANTAIL01 has been hitherto detected only in Australia and Southeast Asia, and *Leucocytozoon* EMSPO05 was known only from black-faced bunting *Emberiza spodocephala* from Russia.

Apart from lineages found in the scarlet rosefinch we detected in this study also six haemosporidian lineages in the other bird species trapped at the study plot (Appendix 1). Among them the *Leucocytozoon* BT2 lineage detected in three bird species was the only lineage shared with the scarlet rosefinch.

We found no association between presence of haemosporidian infections and the expression of condition dependent carotenoid-based colouration of males. The GLM model involving parasite occurrence (*Plasmodium*, *Leucocytozoon*, *Haemoproteus* and the total number of haemosporidian lines found in an individual) as explanatory variables for feather hue was not significant ($F_{4,93}$=0.92, P=0.45), the same was true for feather lightness ($F_{4,93}$= 0.86, P=0.49) and saturation ($F_{4,93}$=0.68, P=0.61). In all cases the simplification suggested the null models as the best models.

In both males and females we furthermore evaluated potential associations between body mass (expressed as body weight/tarsus length) and parasite infection. The model involved haemosporidian genera and the total number of lineages, sex and sex interactions with haemosporidian occurrence and lineage numbers as an explanatory variable for body mass. The MAM only included sex as predictor for body mass ($F_{1,172}$=16.5, P<0.001), suggesting males were lighter for their tarsus length. However, no effect of haemosporidian infections on body mass was detectable (all P>0.15).

Discussion

We detected relatively high prevalence of haemosporidian parasites in the scarlet rosefinch, if compared to other published studies. However, since we studied only one locality our data may not reflect prevalence for the whole large species range. Prevalence differences have been detected among different populations of a single host species (Bentz et al. 2006, Ferrer et al. 2012) or within one population according to landscape features (Wood et al. 2007). Varying host exposure to insect vectors, which can be hardly assessed in migratory birds that can be infected at their wintering grounds, is probably the most important force determining the level of parasite prevalence (Yohannes et al. 2009). In our study, parasite prevalence varied considerably between years. Unfortunately the timescale was too short to reveal regular pattern of oscillations such were shown in the great reed warbler *Acrocephalus arundinaceus* (Bensch et al. 2007).

Since the migratory birds can be infected at breeding, wintering, and also migratory sites, they are supposed to host larger diversity of parasite lineages than the sedentary birds (Smith et al. 2004). This pattern has been demonstrated repeatedly in various species (e.g. Waldenström et al. 2002; Križanauskienė et al. 2006; Bensch et al. 2007; Hellgren et al. 2007; Ortego et al. 2008, Pérez-Tris et al. 2007). Relatively high number of parasite lineages (four *Haemoproteus* and five *Plasmodium* lineages) has been also detected in the paddyfield warbler (Zehtindjiev et al. 2009), which represents similar (European–South Asian) migration system as the scarlet rosefinch. The number of haemosporidian lineages detected in the scarlet rosefinch supports the view of larger parasite diversity in migrant species and contrasts sharply with only 5 lineages detected in the closely related sedentary house finch (*C. mexicanus*) (Kimura et al. 2006). However, it should be noted that most of the scarlet rosefinch lineages were extremely rare. With the exception of SIKIN1 lineage which was detected also in house sparrow (Marzal et al. 2011) all *Haemoproteus* lineages detected in the scarlet rosefinch have relatively narrow host breadth restricted to Fringillidae family. *Leucocytozoon* and *Plasmodium* lineages generally showed wider host range,

in some *Plasmodium* lineages even also comprising non-passerine species.

Tight co-evolution of parasite lineages and hosts that probably prevents parasites to infect sympatric closely related hosts has been reported repeatedly for avian malaria (Reullier et al. 2006, Pérrez-Tris et al. 2007). However, diversity of avian blood parasites was shown not to follow the classical trade-off-hypothesis suggesting that a generalist parasite infecting many hosts should occur at lower prevalence, whilst specialised parasite should rich higher prevalence in the host for which it is specialised (Hellgren et al. 2009). In contrast, Hellgren et al. (2009) showed that both *Haemoproteus* and *Plasmodium* lineages exhibiting the widest host range also occurred at the highest prevalence in a single host species. In concordance with these results, the most frequent *Leucocytozoon* and *Plasmodium* lineages detected in the present study had, indeed, the widest host ranges (*Plasmodium* SGS1 48 host species, *Leucocytozoon* BT2 8 host species). Nevertheless, the most frequent *Haemoproteus* lineage ROFI2, which is the most frequent haemosporidian lineage in the present study and was detected in the scarlet rosefinch also by Križanauskienė et al. (2006) and Bensch et al. (2009), has not been found in any other species, which indicates its strict host specificity.

Haemoproteus lineages SISKIN1 and CCF3 have been previously found only in European sedentary species, which suggests their local transmission to the scarlet rosefinch at the breeding site. We may also speculate that BT2 is transmitted during the breeding season, because it was present in other bird species at the study plot including the sedentary willow tit (*Poecile montanus*). Transmission during the scarlet rosefinch breeding can also be suggested for ROFI1 which was found in *Culicoides* biting midges at the same study plot in 2008 (Synek et al. 2013). On the other hand, the most frequent ROFI2 lineage was not found in haematophagous insects trapped at the same locality, which may suggest its transmission at wintering sites (Synek et al. 2013). Lineages that have been hitherto exclusively detected in Asian bird hosts (*Leucocytozoon* EMSPO05 known from *Emberiza spodocephala*, and *Plasmodium* FANTAIL01 detected in *Acridotheres tristis*, *Dendrocygna javanica* and *Rhipidura rufifrons*) correspond well with the assumed scarlet rosefinch wintering sites in South Asia. Since the EMSPO05 and FANTAIL01 have not been detected in any other European bird hosts, it is highly likely that scarlet rosefinches were infected by those lineages at their wintering sites. However, since avian malaria is far less studied in South Asia than in Europe and Africa we cannot exclude the possibility of winter transmission of other lineages that have been hitherto detected only in Europe and Africa.

Mixed infections of haemosporidian lineages have been repeatedly reported in avian hosts (e.g. Marzal et al. 2008; Valkiūnas et al. 2009b; Asghar et al. 2011; Zehtindjiev et al. 2012). Observed number of mixed infections in the present study closely matched the expected values based on prevalence of individual lineages, which may suggest random pattern of concomitant infections. However, it should be noted that the probability of detecting mixed infections strongly depends on the detection methods. For example, while detection based on manual inspection of electropherograms for double peaks suggested that concomitant infections were less frequent that expected in a long-term population study of great reed warblers (Bensch et al. 2007), closer inspection of partially overlapping dataset using specific primers and highly sensitive quantitative PCR showed on contrary significantly higher frequency of mixed infections than expected (Asghar et al. 2011). Even though we used both manual inspection of electropherograms and specific primer approach, we cannot fully exclude the possibility that we underestimated the frequency of mixed infections, especially in cases of low parasitemia in one of the lineages. On the other hand, in such a case the prevalence of individual lineages would be also underestimated in exactly the same way.

Infection status of birds trapped repeatedly in different years remained mostly unchanged. It may be interpreted either as evidence for a long-term survival of parasites in the host's bloodstream or as evidence for variability in individual host sensitivity to particular parasite lineages and consequent recurrent infections (Hasselquist et al. 2007). While the data showing long-term persistence of haemosporidian parasites in the chronic phase of their life cycle in avian hosts (Valkiūnas 2005, Zehtindjiev et al. 2008) support the former explanation, the known association between MHC alleles and resistance to avian malaria (reviewed in Westerdahl 2007) is consistent with the later explanation. On the other hand, relatively high rates of both *Haemoproteus* and *Plasmodium* lineage losses were also recorded in other bird species (*A. arundinaceus*: Bensch et al. 2007, Hasselquist et al. 2007; *Cyanistes caeruleus*: Knowles et al. 2011). In our study, the number of parasite lineage gains (16) twice exceeded lineage losses (8). However, since the *Haemoproteus* (five losses) and *Leucocytozoon* (two losses) lineages life cycles also involve stages in host internal organs, absence of gametocytes in the host bloodstream does not necessarily imply the full host recovery from the infection. Lineage replacements and the only one detected loss of *Plasmodium* lineage can be probably explained by very low parasitemia bellow the limits of our detection methods. In conclusion, we have not found any strong evidence of complete elimination of parasite lineages and hence our results are compatible with long-time persistence of parasites in bird hosts.

Carotenoid ornaments are generally considered as honest indicators of health (Lozano 1994, Badyaev and Hill 2000,

Vinkler and Albrecht 2010). In the present study, however, we have not found any effect of haemosporidian presence on male coloration and host body mass. In our previous study (Vinkler et al. 2010) based on microscopic examination of blood smears, we have shown that haemosporidian parasites may influence health in the scarlet rosefinch (measured as basophil ratio in peripheral blood). Nonetheless, the present lack of supportive evidence for the haemosporidian influence on body mass is in accordance with number of field and laboratory studies in birds (Sanz et al. 2002; Votýpka et al. 2003, Gibb et al. 2005; Deviche et al. 2005; Palinauskas et al. 2008). It is, thus, possible that the loss of weight due to blood parasites may be only temporary; occurring in acute phase of the infection and birds may recover to their original weight during the chronic phase (Valkiūnas 2005). Studies targeting the effect of haemosporidian parasites on carotenoid-based male coloration are rather scarce and inconsistent. While no effect of haemosporidian parasite presence on male coloration was found in redpoll (*Carduelis flammea*) Seutin (1994), negative correlation between haemosporidian presence and intensity of ornamentation was observed in yellowhammer (*Emberiza citrinella*) (Sundberg 1995). Surprisingly, also positive correlation of parasite presence and male carotenoid ornaments was reported in greenfinch (*Carduelis chloris*) (Merila and Sheldon 1999). Thus, it seems the effect of haemosporidians on carotenoid ornaments can be also rather complex and age dependent, as shown in blue tit (*C. caeruleus*) Hõrak et al. (2001). It should be also noted that the feather ornament of scarlet rosefinches can be influenced mainly during moulting completed during winter. We cannot exclude the possibility that the presence of parasites detected during the breeding season only weakly correlates with the bird infection status in winter. Moreover, acute winter infections that we were unable to detect during their chronic phase in spring may influence the host ornaments. However, this view remains unsupported by the fact that majority of birds trapped repeatedly in different years unchanged their infection status.

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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 and comply with the current law of the Czech Republic.

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

Appendix 1

Table 5 Lineages found in other species trapped at the scarlet rosefinch locality

Host species	No. of individuals	*Haemoproteus*	*Leucocytozoon*
Common Treecreeper (*Certhia familiaris*)	1		
House Martin (*Delichon urbica*)	1		SYBOR8
Chaffinch (*Fringilla coelebs*)	1	CCF1	
Red-backed Shrike (*Lanius collurio*)	2	RB1	BT2
Bluethroat (*Luscinia svecica*)	1		BT2
Willow Tit (*Parus montanus*)	1		BT2
Common Chiffchaff (*Phylloscopus collybita*)	1		
Willow Warbler (*Phylloscopus trochilus*)	1		
Dunnock (*Prunella modularis*)	2	TURDUS2, RB1	
Garden Warbler (*Sylvia borin*)	2		
Common Whitethroat (*Sylvia communis*)	2	CWT3	
Lesser Whitethroat (*Sylvia curruca*)	1		
Blackbird (*Turdus merula*)	1	TURDUS2	

Appendix 2

Table 6 Lineage specificity. Other hosts of the scarlet rosefinch parasite lineages are listed according to MalAvi database. Six lineages found exclusively in the scarlet rosefinch are not included

Lineage		Host species	Geographic region
Haemoproteus	CCF3	*Fringilla coelebs*	Europe
	SISKIN1	*Carpodacus mexicanus*	Europe, North America
		Carduelis spinus	
		Loxia curvirostra	
		Passer domesticus	
	ROFI1	*Carpodacus erythrinus*	Europe
		Coccothraustes coccothraustes	
		Fringilla coelebs	
		Carduelis chloris	

Table 6 (continued)

Lineage		Host species	Geographic region
Plasmodium	SGS1	48 host species	Europe, Asia, Africa, New Zealand
	PADOM02	*Passer domesticus* *Anthus hodgsoni* *Corvus corone* *Luscinia svecica* *Motacilla flava* *Phasianus colchicus* *Emberiza citrinella* *Passer montanus*	Europe, South Asia
	WW3	*Andropadus virens* *Geothlypis trichas* *Quelea quelea* *Phylloscopus trochilus* *Spermophaga haematina* *Quelea quelea* *Phylloscopus trochilus* *Passer montanus* *Passer domesticus* *Cyanocorax yncas* *Luscinia svecica*	Europe, North America, Africa
	TURDUS1	18 host species	Europe, South Asia, Africa
	BT6	*Luscinia svecica*	Europe
	FANTAIL01	*Acridotheres tristis* *Dendrocygna javanica* *Rhipidura rufifrons*	Australia, South Asia
	LK05	*Falco namanni*	Europe
	BT8	*Luscinia svecica* *Ploceus* 2 species *Hypothymis azurea* *Estrilda amandava* *Copsychus* 2 species *Lophura punctuata*	Europe, Africa, South Asia
Leucocytozoon	BT2	*Lanius collurio* *Muscicapa striata* *Phoenicurus phoenicurus* *Phylloscopus trochilus* *Saxicola rubetra* *Sylvia atricapilla* *Sylvia borin* *Hippolais icterina*	Europe, Africa
	BT5	*Luscinia svecica* *Lanius collurio*	Europe
	EMSPO05	*Emberiza spodocephala*	Asia

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ORIGINAL PAPER

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

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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

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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).

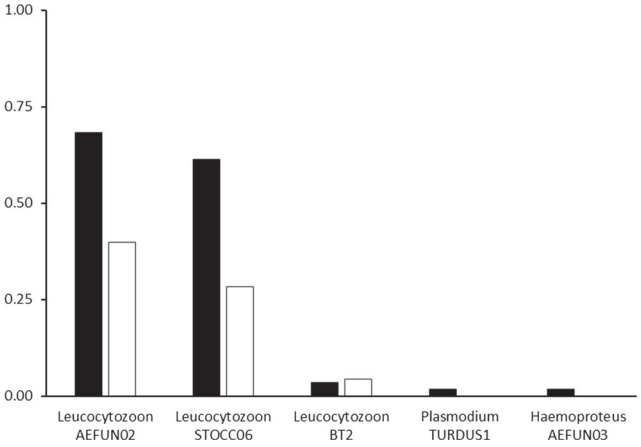

Fig. 2 Prevalence of parasite lineages in adult (*black*) and nestling (*white*) Tengmalm's Owls

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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ORIGINAL ARTICLE

The health signalling of ornamental traits in the Grey Partridge (*Perdix perdix*)

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Abstract Birds express various secondary ornaments that can indicate individual condition and health. Amongst these, red-coloured carotenoid-based ornaments are supposed to be particularly valuable predictors of individual quality, due to their sensitivity to oxidative stress. Nevertheless, melanin-pigmented traits might also signal health and immune functions. Both types of ornaments may be either skin-based or feather-based, each differing in their dynamics. In the present study, we compared the health- and stress-indicating capacity of melanin-based feather ornamentation and putatively carotenoid-based skin ornamentation in a single species—the Grey Partridge (*Perdix perdix*), a vulnerable avian species of the European agricultural landscape. In captive males, we firstly verified the carotenoid content of the red-coloured skin tissue behind the eye by chromatography (HPLC). Secondly, we assessed the individual health status of all males by examining differential leukocyte count, the frequency of immature erythrocytes, malaria prevalence and proinflammatory immune responsiveness to phytohaemagglutinin (PHA). Both the size of the melanin-based ornament and red chroma of the carotenoid-based ornament were related to the heterophil:lymphocyte (H/L) ratio. Hence, in the Grey Partridge, both redness of the skin ornament and area of the feather ornament may serve as honest indicators of individual health and long-term stress. However, the two ornamental components were unrelated to each other, and the directions of their association to the H/L ratio were opposite. We therefore propose that, in this species, larger melanin-based feather ornamentation size is linked to higher levels of stress (possibly caused by more intensive social interactions with other males), while the level of expression of the carotenoid-based skin ornamentation more reliably signals actual individual health status. Our results are potentially valuable from the perspective of Grey Partridge conservation efforts, as they indicate a simple method for assessing individual quality in this species.

Keywords Carotenoids · Condition-dependent · Game bird · Melanin coloration · Phytoheamagglutinin · Plasmodium

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Zusammenfassung Ornamentmerkmale als Signale für den Gesundheitszustand beim Rebhuhn (*Perdix perdix*)

Vögel schmücken diverse sekundäre Merkmale, die die Kondition und den Gesundheitszustand eines Individuums anzeigen können. Aufgrund ihrer Empfindlichkeit gegenüber oxidativem Stress gelten unter diesen rote, auf Karotinen basierende Schmuckmerkmale als besonders wichtig. Dennoch können auch auf Melaninpigment

basierte Merkmale Gesundheitszustand und Immunfunktionen signalisieren. Beide Ornamenttypen treten sowohl in der Haut als auch in Federn auf, wo sie sich hinsichtlich der Dynamik unterscheiden. In der vorliegenden Arbeit verglichen wir die Kapazität von melaninbasierten Gefiedermerkmalen und die vermutlich karotinbasierte Hautfärbung hinsichtlich ihrer Gesundheitszustands- und Stressindikation beim Rebhuhn (*Perdix perdix*), einer gefährdeten Vogelart der europäischen Agrarlandschaft. Von Rebhuhnmännchen aus Gefangenschaft bestimmten wir zunächst mittels Chromatographie (HPLC) den Karotingehalt der rot gefärbten Haut hinter dem Auge. Daraufhin ermittelten wir den individuellen Gesundheitszustand aller Männchen indem wir differenzielles Blutbild, den Anteil unreifer Erythrozyten, Malaria-Prävalenz und die entzündliche Immunantwort auf Phytohämagglutinin (PHA) untersuchten. Sowohl die melaninbasierte Ornamentik, als auch die Rotfärbung der auf Karotin basierten Merkmale waren mit dem Verhältnis Heterophile zu Lymphozyten (H/L) korreliert. Damit können beim Rebhuhn beide Schmuckkomponenten, die Rotintensität der Haut, und Gefiederpartien als ehrliche Indikatoren der individuellen Gesundheit und von Langzeitstress dienen. Allerdings ließ sich kein Korrelation zwischen den beiden Komponenten feststellen, und die Zusammenhänge beider mit dem H/L Verhältnis waren gegenteilig. Wir vermuten daher, dass beim Rebhuhn größere melaninbasierte Gefiederornamente mit höheren Stresswerten in Verbindung stehen (möglicherweise verursacht durch intensivere soziale Interaktionen mit anderen Männchen), wohingegen die Stärke der karotinbasierten Hautornamentik zuverlässiger den individuellen Gesundheitsstatus signalisieren. Unsere Ergebnisse sind potentiell aus der Sicht von Rebhuhnschutzmaßnahmen von Nutzen, da sie eine einfache Methode zur Bestimmung individueller Qualität bei dieser Art aufzeigen.

Introduction

Birds express various colourful ornaments, and, according to the Viability Indicator Hypothesis, these ornaments may signal individual quality and health (Andersson 1994). Health-indicating ornamental traits may be formed either by living skin tissues or dead skin derivates (such as feathers or ramphotheca). Generally, living-tissue ornaments may change their coloration more rapidly depending on actual conditions than feather-based ornaments (Pérez-Rodríguez and Viñuela 2008). Therefore, skin ornaments are supposed to be better predictors of short-term individual quality then plumage. Plumage ornaments, on the other hand, may signal individual health status over a longer period of time (e.g. Dufva and Allander 1995; Figuerola et al. 1999; Vinkler et al. 2012). Avian ornamental traits of both kinds are most commonly pigmented by two classes of pigments, carotenoids and melanins. Carotenoids are typically responsible for red, orange and yellowish coloration, while melanins provide feathers and other skin derivatives with different shades of brown, grey and black (McGraw 2006a). These two classes of colour compounds differ in their chemical structure, origin, metabolism, biological availability and activity (McGraw 2006b; Vinkler et al. 2011). Therefore, it has been suggested that melanin- and carotenoid-based ornaments signal different information regarding individual quality (Badyev and Hill 2000). Carotenoid-based traits may indicate foraging efficiency (Hill 1992; Fitze et al. 2003), parasite levels (McGraw and Hill 2000; Hõrak et al. 2004) and oxidative stress (Alonso-Alvarez et al. 2008; Pérez-Rodríguez et al. 2010), i.e. actual body condition and health. Melanin-based ornaments are usually viewed as traits reflecting dominance (Møller 1987; Senar 1999) or hormonal loads (Evans et al. 2000), i.e. traits under tighter genetic control (Roulin and Dijkstra 2003). Although many studies have reported differences between melanin- and carotenoid-pigmented ornamental traits (e.g. Hegyi et al. 2007; Hill and Brawner 1998; McGraw and Hill 2000; Senar et al. 2003; Tarof et al. 2005), current data do not unambiguously seem to support the idea that carotenoid-based skin ornaments are better predictors of individual condition and health than melanin-based plumage traits. For instance, a recent meta-analysis by Griffith et al. (2006) revealed that there is no difference in condition dependence between carotenoid- and melanin-based ornamental traits. Furthermore, it was shown that feather-based melanins can also be associated with immune function (e.g. Gangoso et al. 2011; Jacquin et al. 2011) and that the direct contribution of carotenoids to health is unclear (Vinkler and Albrecht 2010). Hence, the signalling function of skin carotenoid-based traits and feather melanin-based traits is still intensively debated.

The Grey Partridge is a medium-sized galliform species with a monogamous mating system. This species is a convenient model for evolutionary ecology studies in birds since both sexes express a melanin-based horse-shoe-shaped feather ornament on the breast and a putatively carotenoid-based red skin ornament behind the eye. The Grey Partridge is a common bird of the Eurasian agricultural landscape, but is presently disappearing in many regions (Kuijper et al. 2009). Therefore, in many European countries, Grey Partridges are artificially bred and released to reinforce the local populations (Andersen and Kahlert 2012; Buner et al. 2011; Liukkonen 2006; Vidus-Rosin et al. 2010). However, currently, the survival rate of the released birds is usually low because of predation (Sokos et al. 2008). This might also be due to the poor condition

and health of artificially bred individuals, though the available evidence does not seem to show any difference in body mass index between reared and wild females (Parish and Sotherton 2007). The difference, nonetheless, might lie in some other component of individual quality. Little is known about reliable indicators of individual quality in the Grey Partridge, e.g. ornaments as signals of individual health. These traits have potential utility in conservation because individuals could be easily chosen based on ornaments for further breeding in artificial breeding programs aimed at Grey Partridge reintroduction.

In this study, we investigated the association between melanin-based and presumed carotenoid-based ornamentation, body condition and health-related traits in Grey Partridge (*Perdix perdix*) males. In a manipulative experiment, it was found that the melanin-based feather ornament on the breast does not play an important role in female mate choice, is not age or testosterone dependent and has low heritability (Beani and Dessi-Fulgheri 1995). This questions the signalling function of this trait. The signalling function of the red eye patch has not yet been tested. Although in several galliform species red-coloured skin ornamentation has been shown to be caused by carotenoid pigmentation (e.g. Egeland et al. 1993; Mougeot et al. 2007), the chemical nature of the orange-red eye patch in the Grey Partridge has not yet been determined. To accomplish this, we used high-performance liquid chromatography with photodiode array detection (HPLC). To examine individual health status, we estimated several health-related traits including size, body mass index, differential leukocyte count, the frequency of immature erythrocytes, malaria prevalence and the proinflammatory immune responsiveness to phytohaemagglutinin (PHA). Based on current knowledge, we predicted that, in the Grey Partridge, the skin coloration of the red eye patch would be a better predictor of actual health and condition than the size of the melanin-based feather ornamentation on the breast, and thus might be a better trait to be utilised in Grey Partridge management programs.

Methods

Experimental procedures

Fifty 1-year-old males that were hatched and reared in captivity were obtained from the poultry breeding facility of the Faculty of Veterinary Hygiene and Ecology, University of Veterinary and Pharmaceutical Sciences in Jinačovice, and housed individually in indoor cages (89 cm × 189 cm and 40 cm high). Throughout the experiment, all males were fed ad libitum with wheat grain. Birds were held on an artificial day-length cycle of 12 h dark:12 h light. Before any further investigation, the birds were left undisturbed for 3 days in their cages for acclimation. On 17 April, 2009, all individuals were examined with respect to the following condition-related and ornamental traits. First, approximately 100 µl of blood were taken from the brachial vein of each individual and a blood smear was prepared. Then, weight (measured by spring balance, accuracy 0.5 g; Pesola, Baar, Switzerland) and tarsus length (measured by digital calliper, accuracy 0.01 mm; Kinex, Prague, Czech Republic) were recorded. We examined two ornamental traits potentially important for Grey Partridge mate choice (Beani and Dessi-Fulgheri 1995), the red–orange skin patch behind the eye and the dark-brown horseshoe-shaped patch in the breast plumage. To assess the colour components and area of these ornaments, standard digital images of the breast and both sides of the head were taken of each male by a Perfection V10 scanner (Seiko Epson, Nagano, Japan). All images were taken in a standardised position with grey and colour standard reference swatches equipped with a ruler (grey card GC 18 and colour and grey chart Q 14; Danes-Picta, Prague, Czech Republic) in a dark room. Hence, the distance and light conditions were held constant. Furthermore, the red coloration of the eye patch was measured by an Avaspec 2048 spectrometer with Avalight XE light source (Avantes, Eerbeek, Netherlands). The reflection probe (2 mm diameter) was placed at a perpendicular angle at three points of the ornament on both sides of the head. Finally, birds were tagged by aluminium rings with a unique digit and colour combination. One day later (18 April), we assessed individual proinflammatory immune responsiveness by the PHA skin-swelling test (Cucco et al. 2006; Smits et al. 1999; Vinkler et al. 2010b). Each bird was taken out of its cage and the thickness of the central part of its left wing web (patagium) was measured three times with a pressure-sensitive digital micrometer (Mitutoyo 7301, accuracy 0.01 mm; Mitutoyo, Kanagawa, Japan). Thereafter, each male was injected subcutaneously into the same spot with 1 mg PHA dissolved in 40 µl PBS (product nos. L8754 and D5652; Sigma-Aldrich, St Louis, MO, USA). Then, the individual was placed back in its cage and left calm until the response measurement. After 6 ± 0.5 h, the thickness of the patagium was again measured (three times with the pressure-sensitive micrometer). All thickness measurements were highly repeatable ($r = 0.86$, $n = 48$, $p < 0.001$; Lessels and Boag 1987). The PHA-induced swelling response index was later calculated as the average tissue thickness 6 h after the treatment minus the average thickness before the PHA injection (for usage of the 6 h period, see, e.g., Bonato et al. 2009; Møller et al. 2003; Vinkler et al. 2010b). All treatments and measurements were performed in afternoon hours to minimise time-dependent variation. The research was approved

by the Ethical Committee of the University of Life Sciences in Prague and was carried out in accordance with the current laws of the Czech Republic.

Analyses of ornaments

The digital images of partridge ornaments were analysed using Adobe PHOTOSHOP CS.3 software version 10.0 (Adobe Systems, San Jose, CA, USA). Firstly, colours were standardised according to the 50 % grey, black and white standard of the reference swatch and the image scales were equalised according to the rulers. From these standardised images, areas of the ornaments were measured. Eye patch hue, saturation and lightness were calculated from 3 measurements on both sides of the head (each of area 5 × 5 pixels) randomly distributed over the ornament.

Since the reflectance spectrum of the partridge eye patch showed a bimodal pattern, with one peak in the UV (300–400 nm) and a second one in the red wavelengths (600–700 nm), much like the situation found in the skin ornaments of Red Grouse (*L. lagopus scoticus*; Mougeot et al. 2007), 5 variables of red colour were calculated according to Mougeot et al. (2007) from the spectral measurements: (1) total brightness (sum of reflectance in the interval 300–700 nm), (2) red chroma (reflectance in the interval 600–700 nm, in per cent, relative to total brightness), (3) UV chroma (reflectance in the interval 300–400 nm, in per cent, relative to total brightness), (4) λ RUV (wavelength, λ, at which maximal reflectance was reached in the UV interval 300–400 nm) and (5) λ Rvis50 (the wavelength which corresponds to the reflectance at half the distance between its minimum and maximum in the visible interval). From each measurement, only average values were included in further analyses. We found significant correlations between measurements of: (1) ornament saturation obtained from digital images and spectral red chroma ($r = 0.64$, $n = 48$, $p = 0.004$); and (2) ornament lightness obtained from digital images and total spectral brightness ($r = 0.41$, $n = 48$, $p = 0.004$), i.e. in each case, the two analogous traits measured by two independent techniques provided similar data. This confirms the reliability of the estimates of coloration parameters adopted in this study, and indicates that both approaches are applicable for assessing coloration in the visible wavelengths of the light spectrum. For further analyses, only red chroma and total brightness obtained from spectrometric measurements were used.

Carotenoid identification

On 6 June, 20 males from our experimental group were euthanised, and all ornamental tissue around their left eye was precisely and completely excised and washed several times in ultrapure water to remove residues of blood. The tissue sample was weighed, and then ground thoroughly with a mortar and pestle and transferred to 14-mL glass screw-top test tubes. Carotenoids were extracted with 4 ml of tert-butyl-methylether (TBME; Sigma-Aldrich) in an ultrasonic bath for 1 min. Then, 4 mL of ultrapure water was added and the sample was shaken for 5 min to remove water-soluble pigments. After centrifugation, the upper organic layer containing carotenoids was carefully collected with a Pasteur pipette and the aqueous fraction was extracted two times with the same amount of TBME. The combined organic fractions were evaporated to dryness with a stream of nitrogen, dissolved in 50 μL of a mixture of methanol:acetonitrile (1:1) and applied to HPLC. HPLC separation was performed on a Develosil C30 reverse phase column (RPAqueous C30, 6 μm, 4.6 × 25 mm; Nomura Chemical, Japan) equipped with a C30 precolumn. Methanol (MeOH) and acetonitrile (MeCN) were used as the mobile phase for gradient elution started at 30 % MeCN to 45 % over 30 min, followed by a 10 min wash with 100 % MeCN and 10 min equilibration with the initial 30 % MeCN. Carotenoids were identified using retention characteristics and absorption spectra in the UV and visible light region. Lutein and β-carotene (product nos. X6250 and C4582; both Sigma-Aldrich) were used as external standards and reference compounds.

Haematological assays

In all 50 individuals, the differential leukocyte count and frequency of immature erythrocytes were evaluated. As the procedure has been described elsewhere in detail (Vinkler et al. 2010b) we mention it here only briefly. The air-dried blood smears were stained with Modified Wright-Giemsa Stain (product no. WG128; Sigma-Aldrich) and scanned with an Olympus CX-31 microscope (Olympus, Tokyo, Japan) under ×1,000 magnification to count the proportion of lymphocytes, heterophils, eosinophils, basophils, monocytes and immature leukocytes within a sample of 110–140 leukocytes per smear. As only lymphocytes and heterophils were common leukocyte types in our samples (Table 1), we used the H/L ratio as a common health and stress related parameter for further analyses (see, e.g., Davis 2005; Davis et al. 2008). We also estimated the differential count of immature erythrocytes, a trait predicted to mirror the actual rate of haematopoiesis (see Vinkler et al. 2010b), in 5 randomly chosen monolayer fields photographed at ×100 objective magnification (ca. 500–1,000 cells). The repeatability of the estimate was $r = 0.74$, $n = 10$, $p = 0.003$. Although our experimental birds were kept in a conventional breeding facility complying with all basic hygienic standards, the aviaries of this facility were not protected against flying insects, allowing

Table 1 Summary statistics of the examined immunological traits, condition-related variables and parameters of ornamentation in captive Grey Partridge (*Perdix perdix*) males ($n = 48$)

Variables	Mean	Min	Max	SE	CV (%)
Haematological					
Lymphocyte frequency	49.50	26.28	79.67	1.95	27.35
Immature leukocyte frequency (%)	0.85	0.00	3.88	0.14	111.75
Heterophil frequency (%)	41.39	16.28	64.29	1.96	32.88
Basophil frequency (%)	2.70	0.00	9.02	0.30	76.96
Eosinophil frequency (%)	0.06	0.00	0.80	0.03	335.54
Monocyte frequency (%)	5.49	0.75	21.71	0.57	72.07
H/L	0.98	0.21	2.44	0.08	58.26
Immature erythrocyte frequency (%)	5.36	2.93	11.82	0.25	31.70
Immunological (mm)					
PHA-induced skin swelling	0.76	0.30	1.41	0.04	32.58
Ornaments					
Brightness	221,934.72	139,257.16	339,180.00	5,846.00	18.44
Red chroma (%)	48.99	34.61	61.93	1.06	15.19
UV chroma	17.25	8.83	26.76	0.63	25.43
λ RUV	358.18	346.67	376.00	1.04	2.01
λ Rvis50	559.35	547.51	598.25	1.13	1.42
Size of red patch (mm^2)	57.80	33.90	96.41	1.87	22.70
Size of melanin ornament (mm^2)	1,183.78	396.92	2,276.27	61.41	36.30
Condition					
Tarsus length (mm)	51.43	48.25	57.82	0.28	3.79
Body mass index (g/mm)	6.66	5.27	8.06	0.07	6.85

SE Standard error, *CV* coefficient of variation

natural contact of the birds with haemosporidian parasites. Detection of haemosporidian parasites was done using the nested PCR protocol of Hellgren et al. (2004), which enables distinguishing between infections of *Plasmodium* or *Haemoproteus* and *Leucocytozoon*. All detections were performed twice to reduce the number of false negative results. All positive samples were sequenced with the primer HaemF. Classification of haemosporidian lineages was done according to the MalAvi database (Bensch et al. 2009).

Statistics

For each response variable included in this study (variables of red colour, area of red-based ornament and melanin ornament), a linear model (LM) was created. Body mass index (calculated as individual weight divided by tarsus length to standardise weight to individual size), length of the tarsus, intensity of the inflammatory response to PHA, H/L ratio and immature erythrocyte frequency were included as fixed effects. The significance (set to $p < 0.05$) of particular terms in the models was calculated based on the change in deviance between the full and reduced (null) models. All non-significant terms were removed using a backward stepwise procedure, with the statistic reported for each corresponding to the step when they were removed from the model (Crawley 2002). Two birds were excluded from the analysis because they exhibited extraordinarily high H/L ratios (3.7. and 5.5, median = 0.99 for $n = 50$) indicating severe health problems at the time of the experimental measurements. Therefore, all analyses are based on $n = 48$. All LM models were performed in the software R 2.12.1 (R Development Core team 2008).

Results

Compounds detected in the non-polar extract from eye patch tissue showed the most intensive absorption at wavelengths between 400 and 500 nm and elution between 5 and 20 min. Such wavelengths and retention times are characteristic for oxidized carotenoids. Nevertheless, lutein (retention time 12.0 min) and β-carotene (retention time 15.07 min) were not the dominant carotenoids in the eye patch extract. The most abundant carotenoids found in the tissue were compounds carrying one or more conjugated keto groups.

No significant correlations among the three examined haematological and immunological parameters were found (H/L vs. PHA skin-swelling test: $r = 0.09$; H/L vs. immature erythrocyte frequency: $r = 0.28$; PHA skin-swelling test vs. immature erythrocyte frequency: $r = 0.09$; in all cases $p > 0.05$). Furthermore, there was only a low prevalence of malaria parasites in the blood samples of males. Evidence of infection by *Plasmodium relictum* (lineage SGS1) was found in six birds (12.0 %; $n = 50$) and therefore this trait was not included in further analyses.

We found substantial variation in all measured ornamental traits of Grey Partridge males (Table 1). Though reflectance parameters of the carotenoid-based ornament (i.e. red chroma, brightness, UV chroma) were not related to the ornament size (Table 2), the red chroma correlated negatively with UV chroma, brightness, and λ RUV, and positively with λ Rvis50 (Table 2). Therefore, we used only red chroma (hereafter termed redness) in further LM analyses. We did not find any relationship between the sizes of melanin- and carotenoid-based ornaments (Table 2), indicating that these two ornamental traits are independent of each other.

Our analysis showed that the H/L ratio significantly related to eye patch redness and area of the melanin-pigmented breast ornament (Table 3). Whereas the redness of the eye patch was negatively related to the H/L ratio, the opposite was true for the melanin-based feather ornament (Fig. 1). The area of the eye patch was unrelated to any examined health-related trait (Table 3).

Table 2 Pearson's correlation coefficients (r) between parameters of ornaments ($n = 48$)

	Brightness	Red chroma	UV chroma	λ RUV	λ Rvis50	Area of red patch
Red chroma	−0.61*					
UV chroma	0.63*	−0.89*				
λ RUV	0.04	−0.43*	0.00			
λ Rvis50	−0.36	0.44*	−0.31	−0.28		
Area of red patch	−0.17	0.13	−0.24	0.13	−0.05	
Area of melanin ornament	−0.16	0.11	−0.09	−0.03	0.20	0.19

* $p < 0.05$

Discussion

As in other galliform birds (e.g. Egeland et al. 1993; Mougeot et al. 2007), we found that the ornamental red–orange skin patch behind the eye of the Grey Partridge is pigmented by carotenoids. Although we were not able to precisely describe the carotenoid composition in these partridge ornaments, they mainly carried conjugated keto groups, since their absorption spectra had only one absorption maximum whereas other carotenoids generally have three-peak absorption spectra (Britton et al. 1995).

Our data show that carotenoid ornaments are related to the H/L ratio. Grey Partridge males with paler red eye patches (i.e. low values of red chroma) had higher H/L ratios. Hence, we assume that redder eye patches signal better overall health (e.g. lower pathogen burden and long-term stress). This is consistent with the results of several other similar studies in birds. In the closely related Red-legged Partridge (*Alectoris rufa*), Pérez-Rodríguez and Viñuela (2008) showed a negative relationship between H/L ratio and two traits of carotenoid-based ornamentation in males (eye ring pigmentation and bill redness). Similarly, bill coloration is negatively linked to H/L ratio in male Blackbirds (*Turdus merula*; López et al. 2011) and Zebra Finches (*Taeniopygia guttata*; Birkhead et al. 1998). Contradictory results have been found, however, for some feather-based ornaments. For instance, a positive association between plumage coloration and H/L ratio has been reported in the Great Tit (*Parus major*; Dufva and Allander

Table 3 Results of linear models where red chroma, area of red patch and area of the melanin ornament are response variables

	Red chroma				Size of red patch				Size of melanin ornament			
	Slope ± SE		F	p	Slope ± SE		F	p	Slope ± SE		F	p
H/L	−5.101	1.853	7.858	**0.007**	−3.763	3.540	0.473	0.495	212.384	111.522	4.500	**0.039**
Body mass index	−3.167	2.252	1.880	0.177	3.028	4.302	0.744	0.393	188.972	135.537	2.234	0.142
PHA	4.196	4.322	0.957	0.334	6.061	8.254	0.544	0.465	284.377	260.069	1.245	0.271
Immature erythrocytes	−0.065	0.622	0.011	0.917	1.705	1.187	2.061	0.159	5.817	37.410	0.024	0.877
Tarsus length	−0.581	0.549	1.450	0.235	−0.077	1.049	0.252	0.618	15.424	33.050	0.005	0.943

Statistics for particular explanatory variables were found using a backward stepwise procedure. All statistics correspond to the step when they were removed from the model ($n = 48$, $\Delta df = 1$). Significance at $p < 0.01$ shown in bold

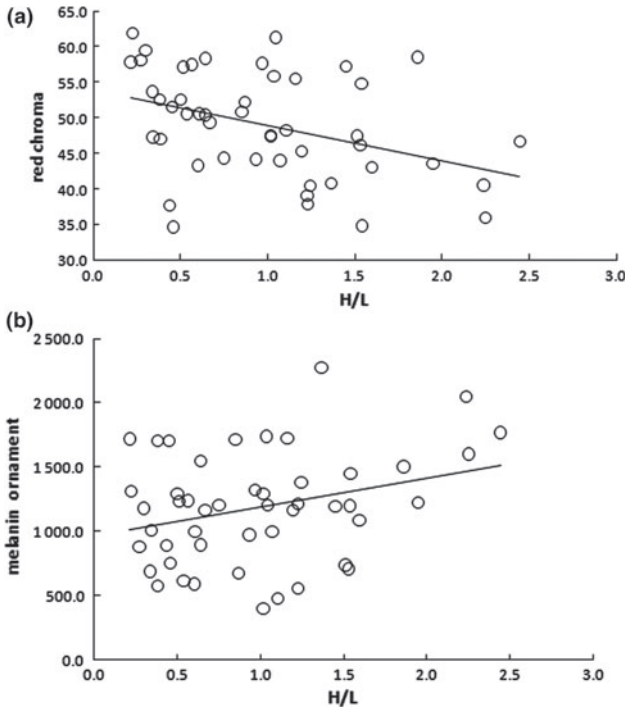

Fig. 1 Associations between H\L ratio and **a** red chroma of the carotenoid ornament and **b** area of the melanin ornament in Grey Partridge (*Perdix perdix*) males ($n = 48$)

1995). Interestingly, this is also consistent with our data in the Grey Partridge, where, in contrast to the carotenoid-based skin ornaments, the melanin-based feather-ornament area was positively related to the individual H/L ratio.

Skin-based and feather-based ornaments differ greatly in their dynamics. While skin may change colouration within a few weeks based on changes of individual health (Mougeot et al. 2010), plumage is stable until the annual moult. Feather-based ornaments, therefore, usually tend to reliably signal long term features of an individual, such as its social status (McGraw et al. 2003). This might explain the difference in H/L linkage between skin-based and feather-based ornaments observed in our study. While the carotenoid-pigmented skin ornament reliably mirrors the actual health state of the male, the melanin-pigmented feather ornament may signal other individual features (e.g. dominance) and these, in turn, could influence individual stress levels (e.g. due to frequent involvement in fights). This is congruent with the results of manipulative experiments in the Grey Partridge reported by Beani and Dessi-Fulgheri (1995). These authors found that males with reduced melanin feather-based ornament size were less involved in antagonistic interactions during the mating period. Thus, largely feather-ornamented individuals should be under higher levels of stress due to their frequent involvement in intrasexual competition, with stress increasing their H/L ratio (Davis et al. 2008). Though social factors were untested in our study, the explanation that the positive linkage between melanin feather-based ornamentation and H/L ratio results from high levels of stress in highly ornamented birds is likely. In addition, this ornamental trait is only weakly related to female-based mate choice (Beani and Dessi-Fulgheri 1995) and hence might play a more important role in male–male competition (reviewed by Jawor and Breitwisch 2003).

The PHA skin-swelling test is the most common test used in avian ecology for assessments of individual pro-inflammatory potential (Vinkler et al. 2010a). Yet, its association with condition-dependent traits including ornaments is still rather controversial. Firstly, the interpretation of this test remains a matter of discussion and intensive research (Martin et al. 2006; Vinkler et al. 2010a, 2012). Secondly, there are many genetic factors that can influence the outcome of the PHA skin-swelling response (Bonneaud et al. 2009) and these often remain unstudied (Vinkler and Albrecht 2011). Thirdly, different types of ornaments (i.e. feather-based, bill and skin-based) vary in their response dynamics to actual individual health (see above). Coloration of the skin as well as plumage is regulated by testosterone (Kimball 2006). Individuals with higher testosterone concentration usually developed brighter ornaments (McGraw 2006c). However, testosterone may have both positive and negative effects on immune function (Cucco et al. 2008; Evans et al. 2000; Mougeot et al. 2004; Navara et al. 2006). Since the carotenoid eye patch of the Grey Partridge is also regulated by testosterone (Beani and Dessi-Fulgheri 1995), high individual variation in testosterone levels together with the delay between ornament expression and the measurement of immunity might explain why neither of the ornaments mirrored the pro-inflammatory potential measured by the PHA skin-swelling test in our captive Grey Partridges.

To conclude, our study confirms that the Grey Partridge skin-based eye patch is pigmented by carotenoids and that this trait might serve as an honest indicator of health in this species. Hence, it may be possibly used by females in mate choice, a hypothesis that so far remains untested. The melanin-based feather ornament showed positive linkage to stress levels, which might result from male–male competition in the pre-breeding season. These results are interesting from the perspective of evolutionary ecology, since they compare the health-signalling potential of a skin-based and feather-based ornament in a single species. Moreover, our findings are also potentially valuable for conservation efforts in this species. Measuring the expression of skin-based ornaments (either by spectrophotometers or from digital images) may be a simple method for screening individual quality in Grey Partridge breeding programmes. This approach might allow

conservation biologists and game managers to choose birds for release based on their quality, and hence increase their survival rates in the wild. It could also lead to more efficient and economical methods of supporting natural populations of this species in Europe. Nonetheless, our study only shows the actual relationship between ornamentation and health. More research is clearly needed to show how stable this relationship is over time and whether selection on its basis could actually improve Grey Partridge survival.

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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.

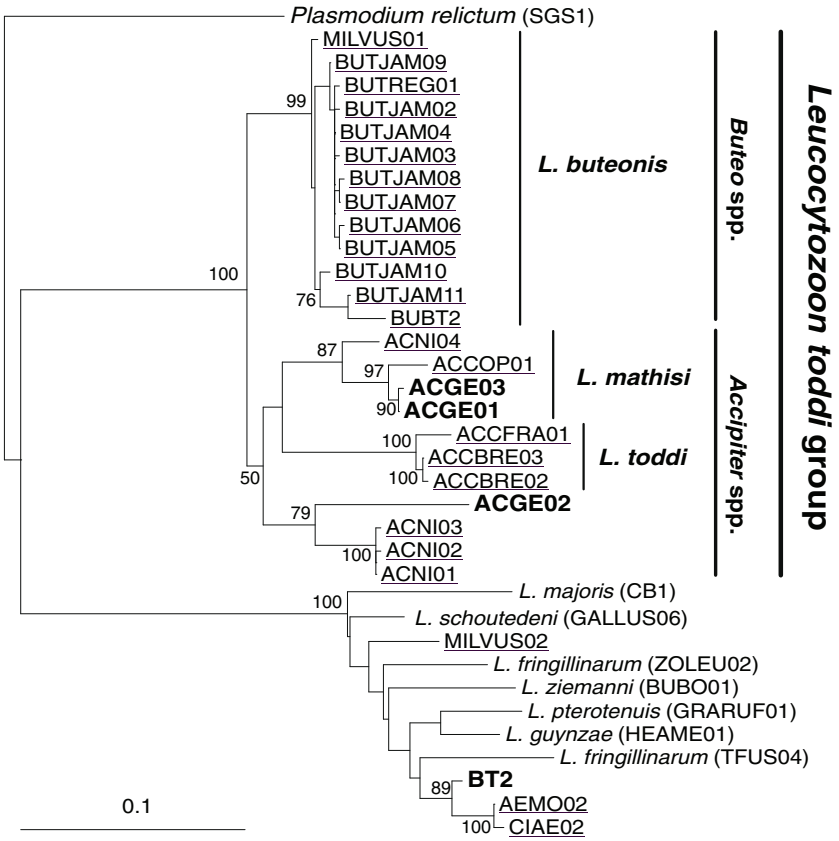

Fig. 1 Maximum likelihood phylogram of *Leucocytozoon* cytochrome b. Lineages previously identified from raptors (Falconiformes) are *underlined* and lineages detected in this study from the northern goshawk (*Accipiter gentilis*) are in *bold. Plasmodium relictum* was used as an outgroup. Newly identified 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)

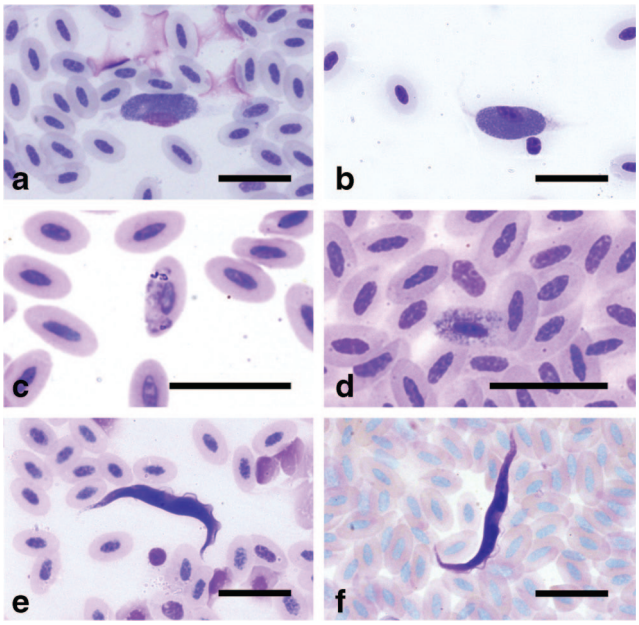

Fig. 2 Light microscopy of Giemsa-stained blood parasites on smears of northern goshawk nestlings and adults. **a–b** *Leucocytozoon* sp.; **c–d** *Haemoproteus* sp.; **e–f** *Trypanosoma* sp.; *scale bars*=5 um

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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