Nest ecology of blood parasites in the European roller and its ectoparasitic carnid fly

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ABSTRACT

Haemosporidian parasites are considered the most important vector-borne parasites. However, vector identity and ecology is unknown for most such host–vector–parasite systems. In this study, we employ microscopic and molecular analyses to examine haemosporidian prevalence in a migratory, cavity-nesting bird, European roller Coracias garrulus, and its nidicolous blood-feeding ectoparasite Carnus hemapterus. This system is unique in that the ectoparasite is confined to a near-closed environment, in contrast to the free-wandering system of haematophagous dipterans such as mosquitoes. Blood film analysis confirms previous works in that Haemoproteus parasites are widely prevalent in adult rollers and belong to a single species, Haemoproteus coraciae. Leucocytozoon sp. and Trypanosoma sp. also are detected in adult rollers at low intensities with this technique. By means of molecular analysis, we report for the first time Plasmodium sp. presence in C. garrulus. Based on PCR results, Plasmodium parasites are relatively less prevalent than Haemoproteus parasites (20% vs. 31%) in rollers. In contrast, haemosporidian prevalences show the opposite trend for Carnus flies: Plasmodium sp. occurrence (62%) clearly predominates over that of Haemoproteus sp. (5%). A comparison between roller and Carnus samples reveals a significantly higher prevalence of Plasmodium sp. in Carnus samples. Insect survey and phylogenetic analysis suggest Culicoides flies as Haemoproteus sp. vectors, which appear to readily transmit the parasite in southern Spain. This study does not find support for Carnus flies to serve as biological or mechanical vectors of haemosporidians. In spite of this, nidicolous blood-feeding ectoparasites, such as...
1. Introduction

Haemosporidian parasites are considered, due to their cosmopolitan distribution and associations with a wide range of vertebrate hosts, the most important vector-borne parasites (Garnham, 1966; Valkiunas, 2005). In addition to direct effects of haemosporidian infection (e.g. Van Riper et al., 1986; Atkinson et al., 1995; Merino et al., 2000), long-term effects of the infection on life span and lifetime reproductive success has recently been demonstrated in birds (Ashgar et al., 2015). While concerns for human and wildlife health rise due to expanding or shifting distributions of vector-borne parasites (see Lafferty, 2009), the knowledge of vector distribution and ecology remains incomplete (e.g. Kraemer et al., 2015).

Despite the popularity of molecular techniques in detecting haemosporidians in hosts and vectors, vectors for most haemosporidians are unidentified (Cleaveland et al., 2001; Valkiunas, 2005), and parasite–vector associations remain an enigmatic aspect of haemosporidian parasite ecology (Atkinson et al., 2008; Kimura et al., 2010; Njabo et al., 2011). For example, a long-term research conducted at a coastal region in northeast Europe suggests that vector competence can be low for some haemosporidians, even if the parasites are ingested by available haematophagous insects (Valkiunas, 2005). It is unclear whether this result is due to the lack of competent vectors or a temporal unavailability of vectors during the breeding period of their avian hosts (Valkiunas, 1984, 2005).

The European roller Coracias garrulus (hereafter roller) is a near-threatened secondary hole-nesting bird, breeding in Southern Spain in burrows excavated by other birds in sandstone cliffs as well as in cavities in bridges and nest-boxes installed on cliffs and trees (Václav et al., 2011). To date, Haemoproteus (Para haemoproteus) coracini, Leucocytozoon eurystomi, Leucocytozoon bennetti, and Trypanosoma avium have been detected in adult rollers during the spring migration period using blood film analysis (Danilewsky, 1889; Valkiunas and Lezhova, 1990; Valkiunas, 1993; Shurulinok and Golemansky, 2002, 2003).

The nest-based ectoparasite Carnus hemapterus is a 2 mm long blood-sucking fly that parasitizes bird nestlings of more than 50 species of 23 families and 10 orders (Grimaldi, 1997; Brake, 2011). After winter diapause, winged adult flies usually emerge in the nests when nestling hosts hatch and emergence continues throughout the whole nestling period (Valera et al., 2003). Adult flies lose their wings as soon as they locate a suitable host (Roulin, 1998). Wingless females of this ectoparasite repeatedly take blood meals from avian nestling hosts in the same cavity and lay egg batches in the nest substrate between feeding bouts (Valera and Židková, 2012). Incubating and brooding adult birds can serve as alternative hosts for C. hemapterus before more suitable nestling hosts hatch in the same cavity (López-Rull et al., 2007; Calero-Torralbo et al., 2013). Carnus flies have never been found, neither as adults nor larvae, on the body of adult birds outside the nest, suggesting that host location occurs exclusively during the winged phase.

Although carnid flies are not usually considered potential vectors for haemosporidians (but see Fitzner and Woodley, 1985; Soler et al., 1999; Martín-Vivaldi et al., 2006), their role as possible biological or mechanical vectors has never been studied. Previously, we reported high infestation rates by C. hemapterus in nestlings of C. garrulus breeding in a semi-arid region in southeast Spain (Václav et al., 2008). The system formed by the cavity-nesting roller and carnid flies is peculiar in that the ectoparasite is confined to a near-closed environment, in contrast to the free-wandering system of mosquitoes and biting midges. Such a spatially constrained feeding habitat suggests intriguing potentials for C. hemapterus co-evolution with both avian hosts and blood parasites.

In this contribution we focus on the nest ecology of blood parasites. Haemosporidian prevalence is scrutinized in this unusual host–ectoparasite system at the spatio-temporal scale of host nest cavities and the host reproductive period. The approach in this study is to examine first the prevalence of haemosporidians for a multi-year sample of adult and nestling rollers. Then, partial gene sequences of mitochondrial and nuclear DNA are analysed for haemosporidians found in adult and nestling rollers and in carnid flies recovered from these hosts. Phylogenetic trees are constructed in order to infer taxonomic relations of haemosporidians detected and their possible dipteran vectors. Finally, within-cavity consistency in haemosporidian prevalence in birds and flies is examined to reveal a potential temporal pattern in haemosporidian prevalence for adult and nestling rollers. We hypothesise that it should be possible to diagnose haemosporidian infection in juvenile birds by PCR-based molecular analysis of their ectoparasitic Carnus flies. If supported, such a diagnostic procedure would be advantageous as it may be a non-invasive indirect method for monitoring haemosporidian infections in birds.

2. Materials and methods

2.1. Site characteristics

The study area (−50 km²) is located within sparsely populated extensive farmland in the Tabernas Desert (Almería, SE Spain, 37°05′N, 2°21′W). The climate is semi-arid with long hot summers and high annual and seasonal variability in rainfall (Lázaro, 2004).

2.2. Taxonomic survey of diptera

A qualitative survey of arthropods, aimed at the identification of putative dipteran vectors, was performed in the nest-boxes of three active roller pairs using sticky cards. About 170 flies were captured during the sampling period of one week in May 2010. Sticky cards were attached to the underside of nest-box lids during the incubation/hatching period of rollers.

2.3. Protocol for blood analysis in adult and nestling rollers — samples examined by microscopic methods

We sampled adult birds by capturing them at the nests during the incubation and hatching periods in the years 2005, 2006 and 2010 (n = 98 for the three years). Blood samples from nestling rollers were obtained only in 2006 (n = 26). Blood films were fixed in absolute ethanol immediately after blood collection and stained within a month with Giemsa for 45 min. For samples collected in 2005 and 2006, infection by intraerythrocytic haemosporidians...
was detected and quantified under oil at 1000× magnification by counting the number of parasites per 2000 erythrocytes (Godfrey et al., 1987). Blood films of adult roller samples collected in 2010 were inspected also for the presence of extracellular parasites (trypanosomes) and some large intracellular parasites (Leucocytozoon spp.) by scanning one-half of the symmetrical smear at 200× magnification (about 300 fields scanned, one-half being chosen at random). Low intensity infections were recorded for Leucocytozoon and Trypanosoma spp. Although most infections with intra-erythrocytic parasites were noted at 200× magnification, we checked another 20 fields at 400× (about 20,000 erythrocytes) in an area with homogeneous distribution of cells in the other half of the smear (the part not scanned at 200×). Subsequently, we used the oil immersion lens to count at least 2000 erythrocytes. When an uneven distribution of parasites or blood cells was noted, replicates of the 20 fields scanned at 400× and cell counts under the oil immersion lens were performed in different areas of the smear (see Merino et al., 1997). For taxonomic determination of Haemoproteus parasites, we calculated the average number of pigment granules and the average nucleus displacement ratio for 225 grown gametocytes obtained from randomly selected blood films of 15 adult rollers (15 gametocytes/roller were examined). The species of Haemoproteus parasites was determined using the taxonomic key by Valkiunas (2005).

2.4. Protocol for roller blood and Carnus fly analysis within roller nests – samples examined by molecular procedures

Blood samples of adult rollers for molecular analysis were obtained by capturing birds at the nests during the incubation and hatching periods in 2011. These periods were chosen because other individuals than putative parents could be captured at the nest during the nestling-feeding period. Nestling rollers, corresponding to the broods of trapped adult birds, were blood sampled twice for molecular analysis during the nestling period in 2011. Nestling rollers usually hatch asynchronously. Hence, the first sampling occurred after hatching of the last chick in the brood when nestlings were 1–2 to 9 days old. The second sampling was conducted one week later (i.e. nestlings were 8–9 to 16 days old). Such sampling schedule was selected to detect potential differences in haemosporidian prevalence during the nestlings' ontogeny while preventing excessive blood sampling and premature fledging of nestling rollers towards the end of their nestling stage. Roller blood samples were stored in lysis buffer prepared according to Jarvi et al. (2002).

One to three feeding female Carnus flies were collected from each nestling roller during the second blood sampling of nestling rollers and stored in absolute ethanol. All blood and Carnus fly samples were stored at 4°C until DNA extraction, which was conducted within a month after sample collection. We did not collect Carnus flies during the first nestling blood sampling because Carnus loads are usually low in very young nestlings (Vaclav et al., 2008). In total, five complete roller families (i.e., 10 adults and 25 (all) nestlings) and 55 flies (10–13 female flies per family) were sampled.

2.5. Dissection of salivary glands and microscopic examination of the glands

The microscopic analysis of salivary glands of Carnus was necessary to evaluate its capacity to transmit haemosporidians. These organs were examined in 33 flies collected from active rollers nests during the breeding period in 2013. Carnus flies were sacrificed by chloroform exposure and salivary glands were quickly removed under stereomicroscope (at 40×). The classical aceto-carmine stain was used for staining of nuclei material (Semichon, 1924). In brief, salivary glands on slides were treated for approximately 1.5 min with a solution of 0.5% carmine in 25% acetic acid and 50% ethanol. Stain solution was removed with a micropipette and then two drops of diluted HCl (1% v/v or less) were used for a quick destain step. Destain solution was removed and biological material in the slide was neutralized with two drops of a solution of sodium bicarbonate (39 g/l) in 35% ethanol. The squash material of salivary glands was finally examined for parasites at 400×.

2.6. DNA extraction, PCR amplification and sequencing

DNA extraction from roller blood and Carnus fly samples collected in 2011 was performed by using the Nucleo Spin Tissue kit (Macherey Nagel). The concentration and quality of extracted DNA was determined by spectrophotometry. The 2× PCR Master Mix (Fermentas) was used for all PCR reactions. Details about the target genes, primers used for DNA amplification, and works describing PCR conditions used in our study are shown in Table 1. Multiple primers were selected and tested in order to analyse their performance as molecular diagnostic tools. Primers employed in PCR and sequencing were capable of amplifying DNA fragments in presumed roller haemosporidians. All roller blood and Carnus fly samples were analysed using the six sets of primers. All specific PCR products were separated by electrophoresis on 2% agarose gels and visualized by staining with ethidium bromide. The specific bands were cut out from the gel, purified by Wizard SV Gel and PCR Clean-Up System (Promega) and sequenced using a commercial service (GATC Biotech AG, Germany). Positive and negative controls were used throughout PCR analyses. Negative samples were tested twice by PCR to reduce the possibility of false negatives. The mtDNA sequence for Haemoproteus sp. was deposited in Genbank and MalAvi databases under the accession number KU297278 and CORCAR01, respectively.

2.7. Phylogenetic and statistical analyses

In order to obtain taxonomic and phylogenetic inference, the sequences obtained were compared to publicly available sequences using a BLASTn search in GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and MalAvi databases (Bensch et al., 2009). Phylogenetic relationships were established based on the analysis of obtained and published sequences of the mitochondrial cytochrome b gene and the nuclear asl gene using MrBayes 3.2.5 software (Ronquist et al., 2012). The program jModelTest 2.1.7 (Darriba et al., 2012) was used to select the best-fit model of nucleotide substitution. Hereby, the GTR + I + IΓ model was selected for both cytochrome b and asl phylogenetic analyses. For each Bayesian analysis, two independent analyses were run with the sampling frequency of 100 and 8–15 million generations. Burn in period was set to default values of 25%. In order to see if Culicoides rather than hypoboscid flies vector Haemoproteus parasites detected in our study, we examined cytochrome b sequences and followed Bukauskaite et al.

Table 1

<table>
<thead>
<tr>
<th>Primer pair name</th>
<th>Target gene</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>HAEMF, HAEMR2</td>
<td>cytochrome b</td>
<td>Waldenstrom et al. (2004)</td>
</tr>
<tr>
<td>L9F, NewR</td>
<td>cytochrome b</td>
<td>Knowles et al. (2011)</td>
</tr>
<tr>
<td>cytBF, cytBR</td>
<td>cytochrome b</td>
<td>Martinisen et al. (2008)</td>
</tr>
<tr>
<td>colf, colR</td>
<td>COX1</td>
<td>Martinisen et al. (2008)</td>
</tr>
<tr>
<td>clpf, clpcR</td>
<td>caseinolytic protease</td>
<td>Martinisen et al. (2008)</td>
</tr>
<tr>
<td>asIF, asIR</td>
<td>adenylsuccinate lyase</td>
<td>Martinisen et al. (2008)</td>
</tr>
</tbody>
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HAEMF/HAEMR2 primers was selected for the cytochrome b analysis. Phylogenetic trees obtained were edited with FigTree 1.4.2 software (Rambaut, 2014). Fisher exact tests were calculated with R software (R Core Team, 2014).

3. Results

3.1. Microscopic and molecular analysis of roller blood samples

Results of the haemoprotozoa microscopic survey in avian blood smears are shown in Fig. 1. *Haemoproteus* sp. infection was found in all adult avian samples, but not in nestling rollers. Based on the gametocyte morphology of *Haemoproteus* parasites, the parasites belong to a single species, *Haemoproteus coraciae* (Fig. 2). Specifically, examining 225 *Haemoproteus* gametocytes for 15 adult rollers, the average width of the gametocyte (±SD), the average number of pigment granules per gametocyte (±SD), and the average nucleus displacement ratio (±SD) was 2.18 ± 0.51 (range 1–4), 7.50 ± 2.44 (range: 2–16), and 0.58 ± 0.18 (range: 0.2–1), respectively. Median infection intensity for this parasite was consistent across sampling years (Fig. 1). *Leucocytozoon* sp. was much more prevalent than *Trypanosoma* sp. in the blood of adult birds, but the intensity of infection for the two taxa was quite low (Fig. 1). *Plasmodium* sp. infection was not recorded by microscopic analysis in any sample.

A total of 60 adult and juvenile roller blood samples obtained in 2011 were tested molecularly for prevalence of haemosporidians using six sets of primers (Fig. 3). The overall prevalence of haemosporidian parasites using this technique was 33% (20/60 samples), with prevalence of 80% (8/10) and 24% (12/50) for the samples of adult and juvenile rollers, respectively. At the individual level, the prevalence of *Haemoproteus* sp. and *Plasmodium* sp. infection in rollers was 31% (11/35) and 20% (7/35), respectively. No multiple infection cases were found by molecular analysis in these samples. While *Haemoproteus* sp. prevailed over *Plasmodium* sp. in adult rollers and 1–9 days old nestlings ($P = 0.023$, Fisher exact test), *Plasmodium* sp. and *Haemoproteus* sp. prevalence did not
differ in older nestlings ($P = 0.247$, Fisher exact test; Fig. 3).

All haemosporidian-positive roller blood samples (8/8 and 12/12 for *Plasmodium* sp. and *Haemoproteus* sp., respectively) were detected by asl primers. HAEMF/HAEMR2, clpc, cytb, and L9/newR primers detected haemosporidians only in a fraction of samples detected by asl primers, and these primers detected *Haemoproteus* sp. only and in blood samples of adult birds only. Finally, col primers did not reveal haemosporidian occurrence in any roller blood sample.

### 3.2. Insect survey and microscopic and molecular analysis of C. hemapterus tissue samples

The taxonomic survey of dipterans revealed five species of three families: Simuliidae, Carnidae, and Ceratopogonidae. Namely, *Simulium velutinum, Simulium petricolum, C. hemapterus, Culicoides circunscriptus* and *Culicoides kibunensis* were trapped in the nests towards the end of the roller incubation period.

The microscopic analysis of salivary glands of 33 female carnid flies collected in active roller nests during the nestling period in 2013 did not reveal the presence of haemosporidians. Based on molecular analysis, haemosporidian prevalence for all flies was 65% (36/55) with *Haemoproteus* sp. detected in 5% (3/55) and *Plasmodium* sp. detected in 62% (34/55) flies (Fig. 3). No Leucocytozoon sp. infections were diagnosed. A case of multiple infection with both *Haemoproteus* sp. and *Plasmodium* sp. was revealed for a single fly. The vast majority (34/34 and 2/3 for *Plasmodium* sp. and *Haemoproteus* sp., respectively) of haemosporidian-positive samples was detected by asl primers. In turn, HAEMF/HAEMR2 primers detected *Haemoproteus* sp. for one fly. Neither haemosporidian parasite was detected for carnid flies by clpc, cytb, L9/newR, and col primers.

### 3.3. Inconsistencies in haemosporidians prevalence

In some cases, nestlings were infected by the same parasite species in the two samplings, whereas others were either positive/negative or infected by a different species in the second PCR test (Fig. 3). *Plasmodium* sp. was detected by molecular analysis in *Carnus* flies in all roller families, but for three of five families, *Plasmodium* sp. was not diagnosed in any bird (be it adult or nestling, Fig. 3). *Haemoproteus* sp. was detected by molecular analysis in carnid flies collected in one roller family, which showed *Haemoproteus* sp. infection only in adult birds (Fig. 3). In contrast, in four of five families, for which *Haemoproteus* sp. was detected by molecular analysis in adult and/or nestling rollers, the haemosporidian parasite was not detected by molecular analysis in carnid flies (Fig. 3).

Analysis of infection rates showed that carnid flies carried *Plasmodium* sp. more frequently than rollers of any age ($P < 0.001$, Fisher exact test). In contrast, *Haemoproteus* parasites in rollers of any age were detected more frequently than in flies ($P = 0.002$, Fisher exact test). In other words, *Haemoproteus* sp. was readily diagnosed in rollers whereas *Plasmodium* sp. was more easily diagnosed in carnid flies.

### 3.4. Genetic similarity of haemosporidians in roller hosts and their carnid ectoparasites

Using BLASTn search, seven samples of adult and five samples of nestling rollers as well as two samples of *Carnus* flies were positive for *Haemoproteus* sp. with asl primers and showed maximum similarity (85–93%) with *Haemoproteus belopolskyi* haplotype P60 (EU254710, Fig. 4). Two of these samples showed maximum similarity (89%) with *H. belopolskyi* haplotype P60 (EU254657) also using clpc primers (Fig. 4). The highest sequence similarity with
Haemoproteus sp. was revealed for two adult roller samples with cytb primers (AY733087, 96%) and for one adult roller sample with L9/newR and ColF/Clpc primers (AF465588, 99%; Fig. 4). As for Plasmodium parasites positive, maximum similarity, based on available sequences of asl gene in GenBank, was revealed with Parahaemoproteus falcatum (AB520157, 69–72%, 19 samples), P. reichenowi (AB519183, 70–71%, 12 samples), and P. mexicanum (EU254674, 69–70%, 11 samples).

The nucleotide sequences of 14 and 42 samples for asl gene that showed maximum similarity with Haemoproteus and Plasmodium parasites, respectively, were analysed to examine sequence similarity within the two genera. The Bayesian phylogenetic analysis does not identify distinct clusters for Haemoproteus parasites, but it appears to differentiate the samples of adult rollers from those of nestling rollers and CorvusFLIES (Fig. 5a). The analogous analysis for Plasmodium sp. reveals that this taxon comprises two different clusters (Fig. 5b). The Bayesian phylogeny of cytochrome b gene sequences and known vector—Haemoproteus spp. associations reveals that Haemoproteus parasites detected in rollers with HAEMF/HAEMR2 primers are vectored by Culicoides rather than hypoboscid flies (Fig. 6).

4. Discussion

We used both microscopy and molecular procedures to examine the ecology of haemosporidians in a host–parasite system consisting of a cavity-nesting avian host and its abundant nidicolous blood-feeding ectoparasite. Molecular analysis of host blood and ectoparasite tissue revealed for the first time that the European roller could be susceptible to Plasmodium sp. infection. In turn, microscopic analysis of blood smears detected three blood parasites already described in rollers, namely, H. coraciae, and Leucocytozoon and Trypanosoma spp. Therefore, the molecular results complement those obtained by blood film analysis in this study but also in the previous studies for this avian species (Danilewsky, 1889; Valkiunas and Iezhova, 1990; Valkiunas, 1993; Shurulin and Golensky, 2002, 2003). Leucocytozoon and Trypanosoma spp. were not diagnosed in rollers by molecular methods, but this could be caused by their very low parasitaemia levels, as determined by microscopy. In other respects, it is important to stress that only asl primers were successful for diagnosing Plasmodium parasites in our study. A recent work suggests that it may be necessary to use a combination of distinct PCR assays to exclude the possibility that mixed infections hinder the detection of specific haemosporidians (Bernotien et al., 2016).

4.1. Haemosporidian diversity in the host—ectoparasite system

The phylogenetic molecular analysis suggests that Haemoproteus parasites detected in nestling rollers and carnid flies are closely related with those obtained from the blood of adult rollers. With respect to nuclear sequences for asl gene, haemosporidian-positive samples showed maximum (85–93%) similarity with Haemoproteus (Parahaemoproteus) belopoloskyi haplotype P60. Concerning the same positive samples, but based on a mitochondrial cytochrome b gene, maximum similarity (99%) was revealed with a sequence of Haemoproteus sp. haplotype 40 (AF465588). Finally, microscopic analysis suggests that a single Haemoproteus species, H. coraciae, occurs in adult rollers breeding in SE Spain, thus corroborating the results obtained for eastern populations of the roller in Bulgaria (Shurulin and Golensky, 2002) and Kazakhstan (Valkiunas...
and Iezhova, 1990).

We are unable to infer taxonomic and phylogenetic relationships for Plasmodium parasites obtained from rollers and Carnus flies. In contrast to Haemoproteus parasites, phylogenetic analysis suggests that rollers can be infected with multiple lineages of Plasmodium parasites, in agreement with data recently published for other bird species in southern Spain (Mata et al., 2015).

In conclusion, we established that Haemoproteus parasites detected in members within every roller family and carnid flies infesting these rollers are all closely related parasites of a single

Fig. 5. Bayesian phylogeny of (a) Haemoproteus and (b) Plasmodium parasites identified in the European roller Coracias garrulus and Carnus hemapterus flies based on 201 bp-long adenylosuccinate lyase sequences. Leucocytozoon sp. was used as outgroup. Sample ID codes N, A, and F mean nestling roller, adult roller, and fly samples, respectively. The first number in sample ID denotes family ID, the second number denotes ID of adults, nestlings and flies within roller families, and the lower case a and b means the first and the second sampling, respectively (e.g. N1-2a means sample of nestling roller #2 of family #1 from the first sampling). Numbers shown in the tree are Bayesian posterior probabilities of the node’s occurrence. The scale bar shows the number of nucleotide substitutions per site.
presumed *Haemoproteus* species. Thus, parasites detected in *Carnus* flies could be used as a genetic indicator of protozoa occurring in their bird hosts. An alternative nested PCR method may be applied to further enhance detection of *Plasmodium* and *Leucocytozoon* spp. (Waldenström et al., 2004).

### 4.2. Haemosporidian associations in the avian host

Using molecular analysis of blood samples, we show that at least two haemosporidians circulate in the blood of nesting roller hosts already during their early ontogeny. Moreover, a concomitant occurrence of highly similar haemosporidians of nesting rollers and *Carnus* flies strongly suggests that haemosporidian prevalence in nesting rollers reflects genuine, not false positive, infection because *Carnus* flies, due to their ecology, could not have obtained the parasite outside the nest. These findings based on PCR analysis differ from blood film analysis results. The latter procedure failed to detect haemosporidians in the blood of nesting rollers. This is in agreement with previous studies showing that blood film analysis can often be insufficient to detect haemosporidians when parasitaemia is low (e.g. Perkins et al., 1998; but see Valkiunas et al., 2006). However, it is also possible that PCR assays detected abortive stages of *Plasmodium* sp. development (Valkiunas, 2011).

Although our work did not aim at addressing the life-cycle patterns of *Haemoproteus* and *Plasmodium* parasites, it indicates that the prepatent period of infection in rollers can be as short as nine days. These results do not conflict with the prepatent periods of *Plasmodium* spp. reported for avian hosts (i.e. 2 days to months, Valkiunas, 2005). However, the presumed prepatent period of *Haemoproteus* sp. in nesting rollers found in this study would be shorter than the 11–12 days lapse reported by Valkiunas and Lezhova (2004) for *H. belopolskyi* in Blackcap *Sylvia atricapilla*. A lower vector availability caused by cooler climate was suggested as a cause for lower haemosporidian prevalence and a longer prepatent period in nestlings in northern Europe (Cosgrove et al., 2008). Warmer climate and a lower availability of avian nestling hosts in late season can favour earlier nestling infection with haemosporidians in late-breeding migratory rollers in southern Europe. Blood analysis of nestlings reared in a vector-free environment (Valkiunas, 2005) or tissue analysis of dead nestlings (Cosgrove et al., 2008) are necessary to confirm PCR-based results suggesting the presumably short prepatent period of both *Plasmodium* and *Haemoproteus* parasites in nesting rollers.

### 4.3. Haemosporidian associations in the ectoparasitic fly

*Carnus* flies have not been previously confirmed as vectors of haemosporidian parasites, and our study indicates that *Carnus* flies cannot serve as a biological vector for haemoproteids or plasmodiids because our survey did not detect haemosporidians in the flies’ salivary glands. However, such results need to be confirmed for a larger sample size. Based on our insect survey in active roller nests and the current knowledge of haemosporidian vectors (Santiago-Alarcon et al., 2012), *Haemoproteus* parasites could be transmitted to nesting rollers by ceratopogonidæ flies (*Culicoides* spp.). Indeed, phylogenetic analysis corroborates this assertion,
because *Haemoproteus coracae* detected in our study clustered with parasites vectored by *Culicoides* and not with hypoboscid flies. Given that we detected only a single *Haemoproteus* species in adult rollers, it is intriguing that a recent work has revealed that none of the *Culicoides* species studied was restricted to a single avian host (Bobeva et al., 2015). It remains to be tested if our results are due to a high degree of host-parasite, host-vector, or vector-parasite specificity. Though it is unclear what insect species could be responsible for *Plasmodium* sp. occurrence in roller families, our study corroborates the importance of endophagous insects in the occurrence of haemosporidians in nesting birds (see Synek et al., 2016).

Remarkably, a different pattern of haemosporidian prevalence in nesting rollers and *Carnus* flies suggests that the timing of parasitaemia for *Plasmodium* and *Haemoproteus* parasites, respectively, can be different during the roller nestling stage. This result is of importance because the timing of parasitaemia is thought to coincide with increased transmission possibilities (Kirkpatrick and Suthers, 1988), mainly in terms of vector abundance (e.g. Mpofu, 1985; Merilä et al., 1995; Mabaso et al., 2005). Thus, our PCR-based results indicate that *Plasmodium* rather than *Haemoproteus* parasites achieve higher transmission during the later nestling stage of rollers.

### 4.4. Consistency in host-ectoparasite-haemosporidian associations

One of the important results of this study is inconsistency in haemosporidian prevalence in roller families and *Carnus* flies corresponding to these host families. Several reasons can be responsible for these discrepancies. First, it is possible that such differences are caused by PCR amplification of DNA of sporozoites or remnants of the parasite’s tissue stages, which might be present in the host circulation. These stages can be dead ends of abortive sporogonic development, but provide templates for PCR amplification (Valkiunas, 2011). Second, template quality could have differed depending on storage solution for roller blood (lysis buffer) and *Carnus* tissue (ethanol) samples. Freed and Cann (2006) reported that a lysis buffer can degrade DNA in blood samples stored in a noncyrogenic stage. Thus, different template quality for roller and *Carnus* samples could have contributed to the prevalence discrepancy. A high sensitivity of *as1* primers, requiring relatively short DNA fragments, suggests that DNA degradation might have affected our PCR results. Third, multiple infection can result in a lower PCR detection rate of specific parasites (Valkiunas et al., 2006). One reason for this can be due to differences in primer affinity between parasite taxa (Perez-Tris and Bensch, 2005). This reason, however, is less likely to explain all cases of inconsistency. For example, PCR analysis using six different primer sets did not detect any parasite in roller nestlings for 40% (2/5) of roller families for which haemosporidian parasites were detected in carnid flies. Fourth, inconsistency in haemosporidian prevalence between *Carnus* flies and nestling rollers can be due to genuine temporal differences in haemosporidian parasite intensity in nestling rollers. Such temporal differences could reflect differences in parasite biology (i.e. mode of reproduction and the necessity of proliferation in host internal tissues) and/or differences in the abundance of biological vectors of individual haemosporidian parasites during the breeding period of rollers. Moreover, differential avian host and/or vector immune responses to individual haemosporidians can also be responsible for temporal differences in haemosporidians prevalence during the nestling period. Further work needs to address if the dynamics in prevalence and intensity of erythrocytic infection differs between *Haemoproteus* and *Plasmodium* parasites in adult and juvenile rollers during the breeding season because acute infection with different haemosporidians can have different fitness effects on their hosts (Hellgren, 2005).

### 4.5. Implications

Although malaria infection has a chronic nature and persists in birds for the lifetime (Atkinson et al., 2008), it usually shows short periods of acute parasitaemia (Valkiunas, 2005). Our molecular results corroborate the idea that *Plasmodium* sp. prevalence in avian hosts can be underestimated if birds are blood sampled at single times and arbitrary time points (c.f. Herman et al., 1966; Valkiunas, 1998; Schrader et al., 2003).

This study indicates that nidicolous haematophagous ectoparasites, due to their unique ecology, are an excellent system to reliably and non-invasively assess the taxonomic structure and temporal dynamics in the occurrence of haemosporidians present at low intensity. This is because carnid flies collect blood from their hosts almost continuously (apart from periods of egg-laying), thereby reducing the effect of circadian variation in haemosporidian parasite intensity in host peripheral blood.

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