High Prevalence and Lineage Diversity of Avian Malaria in Wild Populations of Great Tits (Parus major) and Mosquitoes (Culex pipiens)

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Abstract

Avian malaria studies have taken a prominent place in different aspects of evolutionary ecology. Despite a recent interest in the role of vectors within the complex interaction system of the malaria parasite, they have largely been ignored in most epidemiological studies. Epidemiology of the disease is however strongly related to the vector’s ecology and behaviour, and there is a need for basic investigations to obtain a better picture of the natural associations between Plasmodium lineages, vector species and bird hosts. The aim of the present study was to identify the mosquito species involved in the transmission of the haemosporidian parasites Plasmodium spp. in two wild populations of breeding great tits (Parus major) in western Switzerland. Additionally, we compared Plasmodium lineages, based on mitochondrial DNA cytochrome b sequences, between the vertebrate and dipteran hosts, and evaluated the prevalence of the parasite in the mosquito populations. Plasmodium spp. were detected in Culex pipiens only, with an overall 6.6% prevalence. Among the six cytochrome b lineages of Plasmodium identified in the mosquitoes, three were also present in great tits. The results provide evidence for the first time that C. pipiens can act as a natural vector of avian malaria in Europe and yield baseline data for future research on the epidemiology of avian malaria in European countries.

Introduction

Transmission of avian malaria is highly dependent on the distribution, competence and vectorial capacity of vectors. Despite a recent increase in interest toward dipteran insects as major actors in avian malaria systems [1–4], there is a lack of general knowledge of the role of the vectors in this mosquito-borne disease. Most of the information on the vectorial competence of diverse mosquito species has been reviewed by Huff [5] but natural avian malaria vectors have been mostly ignored until very recently [6–8]. Although many species seem to be competent according to laboratory experiments [6], only a few recent studies have identified natural vectors responsible for avian malaria transmission in different parts of the world, with the notable exception of Europe [1,9–16].

Molecular studies on avian malaria have permitted the investigation of questions concerning phylogeny, phylogeography and life-history evolution of Plasmodium parasites [17–21]. However, the epidemiological success of Plasmodium strongly depends on its life cycle within the mosquito and in the vector’s ability to find suitable hosts for taking a blood meal. Furthermore, asexual reproduction of Plasmodium occurs in vertebrate hosts whereas sexual reproduction takes place in the mosquito, making the vector the definitive host [22]. Mosquitoes are thus expected to play a major role in both avian malaria parasites’ evolution and disease transmission [23,24]. It is therefore of prime interest to deepen the understanding of the interactions between the different actors of this complex host-parasite system under wild conditions, the first step being the identification of the vector in such wild populations.

The aims of the present study were to identify the natural vector of avian malaria in two wild populations of great tits (Parus major) in Switzerland, where the prevalence of infection has been shown to be high [20], and to identify and compare malaria lineage diversity among the bird hosts and the mosquito vectors by means of mitochondrial cytochrome b gene sequencing.

Materials and Methods

Ethical notes

All necessary permits were obtained for the described field studies and the study was approved by the «Conservation de la Faune du Canton de Vaud». No other specific permissions were required for this study and study sites were non-protected, public properties. This study did not involved endangered or protected species. All birds were treated in accordance with the Cantonal Veterinary Authorities of the Canton de Vaud, Switzerland, authorization 1730. Captures of birds were made under license...
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Study sites
The study was carried out in 2006 and 2007 in two populations of great tits (Parus major) breeding in nestboxes. The first population was located in the forest of Dorigny, on the campus of the University of Lausanne (46°31′N; 6°34′E; alt. 400 m). This forest is mainly composed of beech (Fagus sylvatica) and a mixture of large dominant deciduous species like oak (Quercus) and hornbeam (Carpinus). The second study area was located in the forest of Monods (46°34′N; 6°24′E; alt. 680 m). This mixed forest is composed predominantly of beech and spruce (Picea abies) and surrounded by an adler (Alnus glutinosa) forest flooded with stagnant water.

Sampling of mosquitoes
Mosquitoes were sampled from July to September, using both BG-Sentinel traps (BioGents GmbH, Germany) and gravid mosquito traps (Bioquip, California). The first device, using CO2 (dry ice) as a lure, was used to collect host-seeking mosquitoes whereas gravid mosquito traps were intended to capture fed females just before or after oviposition. As egg laying usually takes place five (up to ten) days after blood feeding [25], complete digestion should have occurred at the time of oviposition ensuring absence of remnant bird blood in the digestive tract. As a precaution, however, female mosquitoes were kept alive for 48 hours after trapping before being killed. Mosquito species were identified by morphology in the laboratory.

Sampling of birds
Adult great tits were trapped in their nestboxes during the breeding season by using door traps mounted inside the nestboxes when their nestlings were fourteen days old. A blood sample (20 µl) was then taken by brachial venapuncture and stored at −20°C until molecular assessment of malarial lineages.

Molecular analyses
DNA was extracted from thoraxes of unfed mosquitoes and from adult bird blood samples using the DNeasy tissue extraction kit (Qiagen) according to the manufacturer’s protocol, and was resuspended in 200 µl TE buffer.

Parasite lineages were detected by using a nested PCR method developed by Waldenström et al. [26] from the original protocol [17], amplifying a portion of the mitochondrial DNA (mtDNA) cytochrome b gene. Full details of PCR and sequencing conditions are described in Christe et al. [20]. Sequences were edited and aligned using the program SEQUENCHER 3.0 (Gene Codes, Ann Arbor, Michigan) with additional manual editing. Mitochondrial DNA lineages were identified by comparison with published sequences available on GenBank, and named according to the MalAvi database [27]. Sequences overlapping with less than the 478-bp reference sequence of the MalAvi database were assigned to a precise haplotype name only if they were identical to a reference sequence present in the database. For linkage between mtDNA lineages and morphospecies, we referred to studies combining molecular and morphological approaches [28–30] as well as publications matching cytochrome b haplotypes to a sequence in GenBank associated with a particular morphotype. Sequences were deposited in GenBank (Accession Nos. JQ78276–JQ78282).

Results
Six genera of mosquitoes representing a total of 15 species were captured from the study populations. Among them, nine species (four genera) are mainly or potentially ornithophilous and were analyzed for the presence of Plasmodium and Haemoproteus parasites (Table 1). Only Culex p. pipiens individuals were found to be positive for avian malaria with an overall prevalence of 6.6% (n = 394).

All infected C. p. pipiens originated from the Dorigny forest (26 out of 330 individuals collected, 7.9%), whereas none of the 64 collected in the Monods forest were found to be infected. Proportions of infected birds at both locations were comparable, with 91% of infected birds in the Monods forest and 98% in Dorigny.

Six different Plasmodium lineages were identified among the 26 infected C. p. pipiens, and four among the 54 great tits analysed (Table 2). One lineage of Haemoproteus, known to be transmitted by non-mosquito vectors, was found in a great tit individual.

Discussion
The relatively high prevalence of Plasmodium spp. found in females C. p. pipiens strongly suggests that this species acts as a vector of avian malaria in one of our study populations. C. p. pipiens was infected with six Plasmodium lineages, among which three were also identified in great tits. Haemoproteus spp., transmitted by biting midges of the genus Culicoïdes [31], was present in one great tit individual.

Many species of Culex have been described as vectors of avian malaria parasites and their role as competent vectors in experimental infections has been proven [32]. C. p. pipiens, or the two subspecies C. p. pipiens and C. p. pallens, have been recently reported to be the natural vector of avian malaria in Japan [1,10–14] and North America [2]. Other species of the genus Culex have been described as vectors of avian Plasmodium: C. restuans in North America [2,16], C. (Melanoconion) opossa in Panama [8], C. saltanensis in Brazil [33], C. sasai in Japan [14], C. quinquefasciatus in Japan [12] Hawai’i [34] and Mexico [15], C. neavei, C. perfidiosus, C. pipiens, C. quinquefasciatus, C. annulirostris in the southwest Pacific islands [9]. The results of the present study suggest therefore for the first time the role of Culex

| Table 1. Analyses of ornithophilous mosquito species per study location. |
|-----------------------------|-----------------------------|-----------------------------|
| Species                    | Dorigny                     | Monods                      |
| N (+)  %                    | N (+)  %                    | N (+)  %                    |
| Anopheles maculipennis     | -                            | -                            | 2 (0) 0 2 (0) 0 |
| Culex hortensis            | -                            | 0                            | - 1 (0) -  |
| Culex pipiens              | 330 (26) 7.9                | 64 (0) 0 394 (26) 6.6       |
| Culex torrentium           | 1 (0) 0                     | -                            | 1 (0) -  |
| Culiseta alaskaensis       | 1 (0) 0                     | 24 (0) 0 25 (0) 0           |
| Culiseta anulata           | 5 (0) 0                     | 3 (0) 0 8 (0) 0             |
| Culiseta fumipennis        | -                            | 2 (0) 0 2 (0) 0             |
| Culiseta moritans          | -                            | 31 (0) 0 31 (0) 0           |

The number of screened specimens (N), the number of positive samples (+) as well as the prevalence (%) is given. doi:10.1371/journal.pone.0034964.t001
mosquito thoraxes. One potential problem to this approach is that whereas the mammophilic Anopheles plumbeus listed in Table 1, some mammophilic and opportunistic species are trapping at the canopy level. Apart strict ornithophilic species showed a spatial preference for canopy level [38]. In the present study, gravid mosquito traps were placed at ground level whereas sentinel traps for Japan [1]. In laboratory conditions, P. relictum develops in more than 20 mosquito species including C. pipiens [32] belonging to six genera and has a large bird host range including more than 300 species from different orders and a worldwide distribution [6]. P. polare (SW2) was well represented both in the bird and mosquito samples. According to Valkiunas [6], this parasite has been recorded among 22 bird species in all zoogeographical regions except Antartic and Australia. However, to our knowledge, the present study is the first to report its presence in C. pipiens. Another common lineage, TURDUS 1, assigned to the morphospecies P. circumflexum, has also been found both in great tits and, for the first time, in C. pipiens. This morphospecies has been recorded in over 100 bird species across all regions except the Antartic [6]. Finally, lineage SYAT05, assigned to Plasmodium vaughani, has been found in more than 200 Passeriformes as well as other bird orders [6]. C. pipiens and C. restuans have been reported as vectors for this lineage [2].

Knowledge on temporal and spatial patterns of parasite transmission is essential to understand the selective pressure imposed by parasites on their hosts. For the first time described as a natural vector of avian malaria in Europe, Culex pipiens seems to play a major role in the transmission of a high range of Plasmodium morphospecies. The higher diversity of lineages of Plasmodium found in C. pipiens compared to those found in great tits also

| Lineages | Reference | GenBank accession no. | Morphospecies | No. of infected birds | No. of infected mosquitoes |
|----------|-----------|-----------------------|---------------|-----------------------|---------------------------|
| SGS1     | [17]      | AF495571              | Plasmodium relictum | 33                     | 6                         |
| GRW11    | [35]      | AY831748              | P. relictum     | 0                     | 2                         |
| P5       | [36]      | DQ838991              | P. relictum     | 0                     | 2                         |
| TURDUS1  | [37]      | AF495576              | P. circumflexum | 7                     | 5                         |
| SW2      | [37]      | AF495572              | P. polare      | 13                    | 5                         |
| SYAT05   | [19]      | DQ847271              | P. vaughani    | 0                     | 6                         |
| PARUS1   | [17]      | AF254977              | Haemoproteus majoris | 1                     | 0                         |
| TOTAL    |           |                       |                | 54                    | 26                        |

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suggestions that C. pipiens is a natural avian malarial vector for several other bird species. Furthermore, the presence of some of these Plasmodium lineages in bird species sampled in Africa [40] suggests that in these migrating species, infection may also occur in Europe.

Most of the malarial studies to date have used non-natural systems (i.e. hosts and parasites that would not encounter one another in nature). Using vertebrate/mosquito/haemosporidian model systems from natural populations of wild species [31–43] may provide powerful insights into the complex interactions and constraints affecting the relationship between hosts and parasites.

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Author Contributions

Conceived and designed the experiments: OG PC. Performed the experiments: KI PC OG LF JI. Analyzed the data: KI LF OG PC. Wrote the paper: OG PC LF.
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