Parasitol Res (2012) 111:637–645
DOI 10.1007/s00436-012-2880-3

ORIGINAL PAPER

Avian malaria infections in western European mosquitoes

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Received: 3 February 2012 / Accepted: 23 February 2012 / Published online: 20 March 2012

Abstract In the complex life cycle of avian malaria parasites (*Plasmodium* sp.), we still have a poor understanding on the vector–parasite relationships. This study described the community of potential avian malaria vectors in four Portuguese reedbeds. We tested if their geographical distribution differed, and investigated on their *Plasmodium* infections. The mosquitoes' feeding preferences were evaluated using CO_2, mice, and birds as baits. The most abundant species were *Culex pipiens*, *Culex theileri*, and *Ochlerotatus caspius* (and, in one site, *Coquillettidia richiardii*). *Plasmodium* lineages SGS1 and SYAT05 were found in unengorged *Cx. pipiens* and *Cx. theileri*, respectively, suggesting that these mosquitoes were competent vectors of those lineages. The species' abundance was significantly different among sites, which may help to explain the observed differences in the prevalence of SGS1. At the study sites, SGS1 was detected in the most abundant mosquito species and reached a high prevalence in the most abundant passerine species. Probably, this parasite needs abundant hosts in all phases of its cycle to keep a good reservoir of infection in all its stages. *Cq. richiardii* showed an opportunistic feeding behavior, while *Cx. pipiens* appeared to be more mammophilic than previously described, perhaps because the used avian bait was not its preferential target. In one of the study sites, mosquitoes seem to be attracted to the Spotless Starling *Sturnus unicolor*, an abundant bird species that may be an important local reservoir of avian malaria infections. To our knowledge, this is the first report of detection of avian *Plasmodium* DNA from European mosquitoes.

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Introduction

Malaria parasites (protists of the genus *Plasmodium*) are known to be transmitted by mosquitoes to birds, reptiles, and mammals, including humans. Avian malaria affects most investigated bird species, both wild and domestic (Valkiūnas 2005). The parasites' transmission rate is a function of the vector abundance, host specificity, and ecological requirements (van Riper III et al. 1986). To fully understand parasite evolution and transmission, knowledge of all three components of the vector-vertebrate host-parasite system is essential. Yet little is known about parasite–vector associations in the wild, although predictions are that most parasite species should be vector generalists (Gager et al. 2008), not tightly coevolved with determined vector species (Kimura et al. 2010; Njabo et al. 2011).

Avian malaria parasites reproduce inside the body of female mosquitoes, most frequently of the genus *Culex*, but also *Aedes*, *Anopheles, Coquillettidia*, and *Culiseta* (Atkinson and van Riper III 1991; Njabo et al. 2009; Valkiūnas 2005). A mosquito becomes infected by feeding on the blood of an

infected bird. It will afterwards become infective to other birds if the parasite is able to leave the mosquito's midgut, reproduce, and migrate to the mosquito's salivary glands to be injected into the bloodstream of the donor of the next blood meal (Atkinson and van Riper III 1991; Valkiūnas 2005). If a parasite is able to complete all these stages of its life cycle inside a mosquito, that mosquito species is said to be the parasite's vector. Each *Plasmodium* species may use a number of different mosquito species as vectors (Valkiūnas 2005), although the specific list of vectors for most parasite species has not been determined yet.

For their high humidity and low salinity, reed beds are ideal habitats for mosquito development (Cox 1993). However, differences in habitat and environmental characteristics can cause even close-by locations to have rather different mosquito communities, which in turn can influence the transmission dynamics of the parasites that they vector (Shurulinkov and Ilieva 2009; Sol et al. 2000). This study characterized the mosquito community of four Portuguese reed beds that are important for birds. Regarding those mosquitoes as possible vectors of avian malaria, we aimed to identify avian *Plasmodium* lineages in them and to investigate their host preferences. We tested whether vector communities were similar among sites and if avian malaria prevalence in those sites could be a result of local patterns of vector abundance.

Materials and methods

Study area

This study took place in four Portuguese marshes: Taipal (40° 11′N, 8°41′W), Tornada (39°26′N, 9°08′W), Santo André (38° 4′N, 8°48′W), and Vilamoura (37°04′N, 8°07′W). Santo André is a brackish water coastal lagoon, while the others are coastal freshwater marshes. The still waters of these study sites are good breeding areas for mosquitoes, which were abundant from May to September (personal observation). The vegetation is dominated by vast extensions of reed (*Phragmites australis*) and harbor many bird species of reed bed passerines, ducks, and waders. During the mosquito season, these are breeding sites for resident passerines such as the Cetti's Warbler (*Cettia cetti*) and for migratory species such as the Reed Warbler (*Acrocephalus scirpaceus*). They are also important refueling areas for birds passing during their migration, such as the passing migrant Willow Warbler (*Phylloscopus trochilus*). The surrounding areas are agricultural lands, pastures, and open forests of White Willow (*Salix alba*), Stone Pine (*Pinus pinea*), and Common Alder (*Alnus glutinosa*). Since these marshes gather potential hosts and potential vectors, they have a strong potential for the transmission of avian malaria, which has been proven to happen locally (Ventim et al. 2012).

Field work

From June to October 2008, 19 sampling sessions took place on isolated nights, from sunset to sunrise in Taipal, Santo André, and Vilamoura. From July to September 2009, another 13 mosquito surveys took place only in Tornada. Adult mosquitoes were captured on the marshes' shores with Centre for Disease Control (CDC) light traps (Miniature Downdraft Blacklight UV Trap, John W. Hock Company, Gainesville, FL, USA), baited with CO_2 (dry ice). The traps were set from dusk to dawn, during nights with no rain and light wind. These surveys were always followed by sampling sessions for passerines, which allowed the characterization of the local avian malaria community during these periods (Ventim et al. 2011, 2012).

In order to test the mosquito feeding preferences, eight capture events were made using animal baits on Tornada in August and September 2009, on eight of the nights with mosquito abundance surveys. Apart from previously referred trap baited only with CO_2, two other CDC light traps were set from dusk to dawn, each baited with CO_2 and a caged house mouse (*Mus musculus*) or a caged Bengalese Finch (*Lonchura domestica*). These two animal baits were chosen because they have similar weights (10–15 g), as the bait's weight may influence mosquito attraction (Suom et al. 2010). Both the house mouse and the Bengalese Finch were domestic animals previously kept in cages, which should minimize their stress; they were totally protected with mosquito nets, so that they could not be bitten. The traps were located 10–15 m from each other. The position of the traps and the baits changed from survey to survey. All the collected insects were inactivated by refrigerating them at 4°C and then kept frozen at −20°C until they were analyzed.

Laboratory work

The mosquitoes were identified under a stereomicroscope, on a chill table, using the identification keys of Ribeiro and Ramos (1999) and Schaffner et al. (2001). Males were discarded, and unengorged females were individually frozen at −20°C or pooled by species and by trapping date, in pools of no more than 50 individuals, and homogenized in 2 ml of BA-1 diluent (Lanciotti et al. 2000). Engorged females were individually examined. After their abdomen was separated from the thorax, head, and salivary glands, their blood meals were analyzed for the identity and infection status of the blood donor.

Total DNA was extracted with ammonium acetate from the unengorged mosquito samples. To check if the extracted samples were in good condition to undergo polymerase chain reactions (PCR), the mosquito DNA was amplified. The invertebrate-specific primer pair LCO1490 and HCO2198 (Folmer et al. 1994) was used to amplify a 658-bp fragment

of the mitochondrial gene cytochrome oxidase c subunit I. The protocol was adapted from Whiteman et al. (2006): each PCR tube had a total volume of 25 μl and contained 1.25 μl of 10X PCR buffer, 1.45 μl of 25 mM $MgCl_2$, 1.5 μl of each primer (diluted to 100 μM), 0.8 μl of 100 μM deoxynucleoside triphosphates (dNTPs), 0.75 μl of AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA), 2 μl of BSA (bovine serum albumin), 13.7 μl of sterile dH_2O, and 2 μl of template mosquito DNA. The thermal profile started with 3 min of denaturation at 94°C, followed by 35 cycles of 94°C for 30 s, 45°C for 30 s, 71°C for 60 s, and ended with final a extension step at 72°C for 10 min. The resulting products were run in 2 % agarose gels stained with ethidium bromide for band detection under UV light. Only the samples that produced detectable bands were scanned for malaria infections.

The abdomens of engorged females were cut with sterilized disposable scalpel blades and were individually analyzed for the identity and infection status of the blood donor. Blood-meal identification by PCR is successful as long as the blood-meal residue in the abdomen is still distinguishable by eye (Kim et al. 2009a). DNA was extracted from each abdomen with a silica-based method, the QIAamp DNA Micro Kit (Qiagen, Netherlands), following the manufacturer's protocol. The blood donors were identified by the amplification of their cytochrome b with two primer pairs: Ma-1_F/Ma-1_R was specific for mammals (Ngo and Kramer 2003) and Avi-F/Avi-R was targeted at avian samples (Cicero and Johnson 2001). PCR reactions contained 1.4 μl DNA, 0.5 μl of each primer (10 μm), 5 μl Phusion® Flash High-Fidelity PCR Master Mix (Thermo Fisher Scientific, USA), and 2.6 μl of dH2O. It included one cycle of 30 s at 98°C, 38 cycles (3 s at 98°C, 10 s at 59°C, and 12 s at 72°C), and a final extension at 72°C for 5 min. The results were verified through electrophoresis in agarose gels. Successful amplifications were precipitated and sequenced. The results were edited and assembled in Geneious 5.5 (Biomatters, New Zealand) before performing a BLAST query to identify the most likely species match.

Hemoparasite infections in the mosquito samples were detected by nested PCR (Waldenström et al. 2004), as was also done to the passerine blood samples (Ventim et al. 2011). The used primers were specific for *Haemoproteus* and *Plasmodium* spp.: HaemNF/HaemNR2 (Waldenström et al. 2004), followed by HaemF/HaemR2, which amplified a portion (479 bp) of the parasite's mitochondrial cytochrome b gene (Bensch et al. 2000). Each reaction had a total volume of 25 μl and included 2 μl of template DNA approximately at 25 ng/μl, 2 μl of BSA, 2.5 μl of 10X PCR buffer, 1.1 μl of MgCl2 at 25 mM, 2.5 μl of dNTPs (400 mM of each), 1 μl of each primer at 10 mM, and 0.1 μl of DNA polymerase at 5 Ud/μl. The thermal profile had an initial denaturation step at 94°C for 3 min, followed by cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 45 s, and a final elongation step at 72°C for 10 min. The preamplification PCR ran for 20 cycles, and the final PCR ran for 35 cycles (Waldenström et al. 2004). Contaminations were ruled out by including a negative control (water) per each 24 samples during extraction and another negative control per each 11 samples during PCR. The final amplification products were run in a 2 % agarose gel. Negative results were confirmed by a second nested PCR. Samples showing positive amplification were precipitated with ammonium acetate and ethanol and sequenced using primer HaemF. The obtained sequences were aligned and edited with BIOEDIT (Hall 1999) and compared with the sequences stored at GenBank and the MalAvi database (Bensch et al. 2009) for identification of the parasite's lineage.

Data analysis

Differences of mosquito distribution between sites were tested with a Chi-square test (H_0: the distribution of mosquito species is the same in all sites). Mosquito abundance was measured as the average number of catches per night, thus correcting for the different number of surveys among sites. The same test was used to look for geographical differences in malaria prevalence in juvenile birds of the local communities of passerine species, as scored by Ventim et al. (2011). The use of juvenile birds guarantees that all infections were transmitted locally (Ventim et al. 2012; Waldenström et al. 2002) and rules out the relapses of old infections that may occur in older birds (Valkiūnas 2005). Therefore, the prevalence among juveniles should approach parasite incidence (number of new cases per cohort).

For each mosquito species and each site, the monthly abundance was measured as the maximum catch of unengorged females per trap during that month. The maximum trap catch is assumed to best represent population size, while the submaximal samples in the same period result from a reduced activity rate, perhaps due to less optimal weather conditions. Changes in population size over time are therefore best represented by the trend in maximum trap catch over time (Baylis et al. 1997). In the present work, we have arbitrarily defined that period as monthly. A Spearman correlation coefficient was calculated between the monthly mosquito abundance and the monthly malaria prevalence in juvenile birds of each site.

To evaluate the infection rate of the mosquitoes, the minimum infection rate (MIR) of each mosquito species was calculated (White et al. 2006). When a mosquito pool gave a positive result for avian malaria, it was assumed that there was at least one infected individual in the pool. So, for each species, the MIR (percentage) is as follows:

$$\mathrm{MIR} = \text{Number of PCR positive samples} / \text{Total number of analyzed mosquitoes} \times 100$$

The feeding preference of each mosquito species from Tornada was analyzed by comparing the number of mosquitoes captured with each bait (CO_2, house mouse, and Bengalese

Finch), using the nonparametric Kruskal–Wallis analysis of variance (Statsoft 2011).

Results

Mosquito abundances

The traps baited only with CO_2 collected 2,702 unengorged female mosquitoes belonging to 13 species (Table 1). Only the mosquitoes from the complexes *Anopheles claviger* s.l. and *Ochlerotatus detritus* s.l. could not be identified to the species level, because these two species complexes contain more than one species in Portugal that are indistinguishable with the used protocol (Ribeiro and Ramos 1999). Other species belonging to species complexes could be confidently identified, because the complex does not comprise any other species in Portugal and/or in the studied habitat (Ribeiro and Ramos 1999). These were *Anopheles atroparvus* (from the *Anopheles maculipennis* s.l. complex), *Culex perexiguus* (belonging to *Culex univittatus* s.l.), and *Culex pipiens* s.s. (from the *Cx. pipiens* s.l. complex, although this may include the subspecies *Cx. p. pipiens* and *Cx. p. molestus*).

In the 19 sampling sessions of 2008, 2,038 female mosquitoes of 13 species were captured (Table 1). The most common species overall, and also in each of the sampling sites, were *Ochlerotatus caspius, Cx. pipiens*, and *Cx. theileri* (Fig. 1a–c). In 2009, 13 comparable surveys (baited with CO_2 only) in Tornada contained 664 female mosquitoes of six species (Table 1). The most common species were *Cx. pipiens* and *Coquilletidia richiardii* (Fig. 1d), while *Oc. caspius* was completely absent from the collected samples.

The observed differences in abundance between sites were statistically significant ($\chi_9^2=234$, $p<0.001$) for the four main mosquito species (*Cq. richiardii*, *Cx. pipiens*, *Cx. theileri*, and *Oc. caspius*). *Cx. pipiens* and *Cx. theileri* were more abundant in Taipal (and *Cx. pipiens* also in Vilamoura), while *Oc. caspius* was more abundant in Santo André, and *Cq. richiardii* was the most abundant in Tornada (Table 1). In the local communities of juvenile birds (Table 2, from Ventim et al. 2011), there were significant differences in overall malaria prevalence between sites ($\chi_3^2=10.63$, $p=0.014$); these should be due to the differences in the most abundant lineage, SGS1 ($\chi_3^2=9.72$, $p=0.021$), which is more prevalent than expected in Taipal and Santo André (Table 2). However, the monthly prevalence of avian malaria infections of juvenile birds did not seem to be correlated with the monthly abundance of any mosquito species or with the monthly overall mosquito abundance in each site (Fig. 1); for all tested correlations, $0.00<$ Spearman's $r<0.19$, $p>0.10$.

Malaria infections

Only four unengorged mosquito pools were positive for malaria: two of *Cx. pipiens* (captured at Santo André in June and September 2008), one of *Cx. perexiguus* (Santo André, September 2008), and one of *Cx. theileri* (Taipal, September 2008). Of these four, only two parasite lineages could be sequenced and identified: one infection by *Plasmodium* SYAT05 in *Cx. theileri* (in a pool of 50 females from September 2008) and one by P. SGS1 in *Cx. pipiens* (in a pool of 13 females). The MIR for these mosquito species was of 0.91 % (1/11) for *Cx. perexiguus*, 0.04 % (2/565) for *Cx. pipiens*, and 0.03 % (1/372) for *Cx. theileri*.

Table 1 Total number of unengorged female mosquitoes caught with a CO_2 bait per site, in 2008 (Taipal, Santo André, and Vilamoura) and 2009 (Tornada)

	Taipal 7 surveys	Santo André 7 surveys	Vilamoura 5 surveys	Tornada 13 surveys	Total
Anopheles atroparvus	8	1		1	10
Anopheles algeriensis		35	10		45
Anopheles claviger s.l.	4	1		22	27
Anopheles plumbeus	1				1
Coquilletidia richiardii	2	1	23	444	470
Culex modestus			1		1
Culex pipiens	322	479	160	192	1,153
Culex theileri	195	348	36	1	580
Culex perexiguus	2	13	9		24
Culiseta annulata	1	5		4	10
Ochlerotatus caspius	36	335	8		379
Ochlerotatus detritus s.l.		1			1
Uranotaenia unguiculata		1		1	
Total	571	1,220	247	664	2,702
Nr. Species	9	11	7	6	13

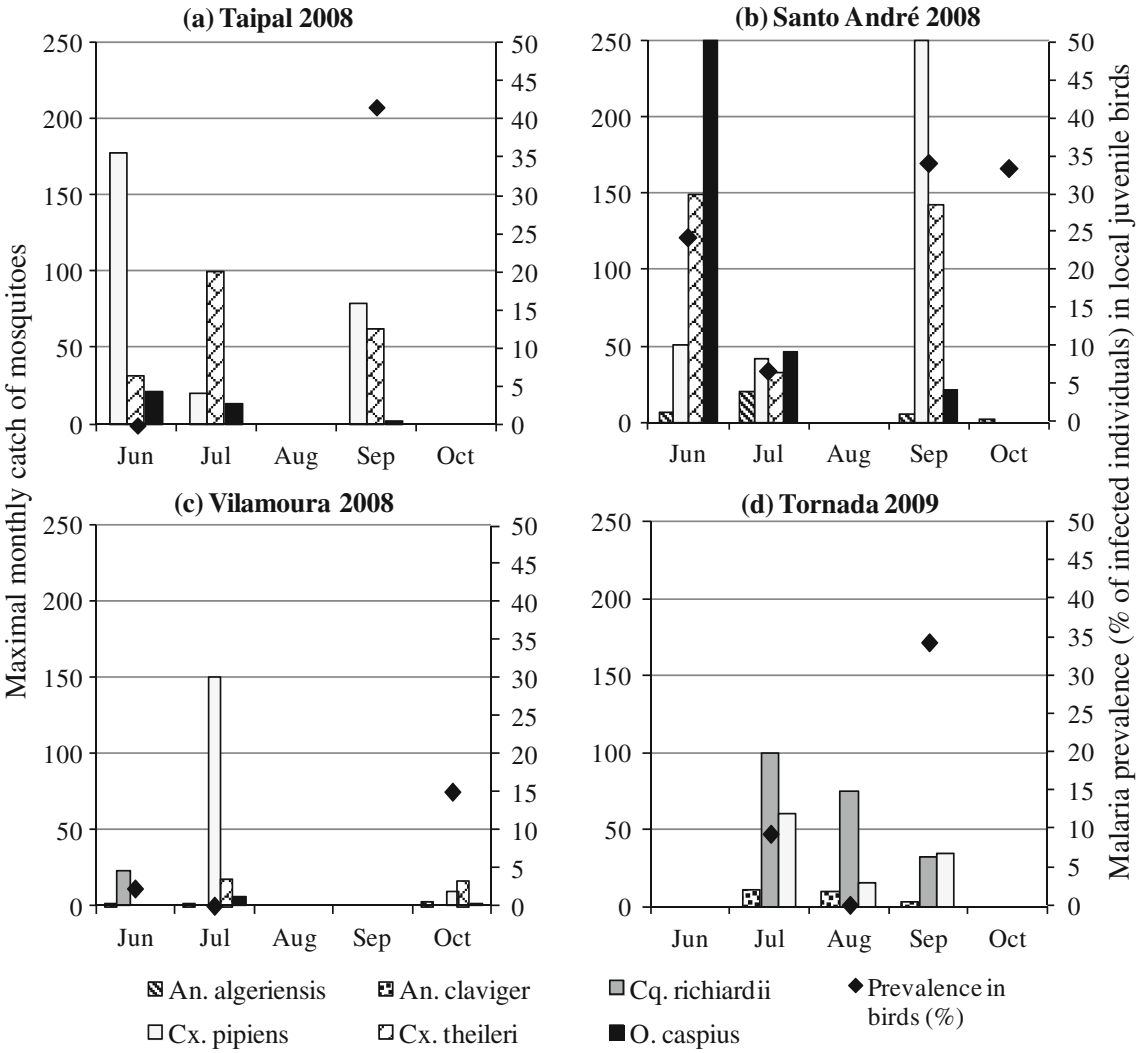

Fig. 1 Monthly abundances of mosquitoes (*bars* are the species that comprise more than 1 % of the site's captures) and monthly malaria prevalence in juvenile birds per site (*black diamonds*). *Left axes* measure mosquito abundance (maximum catch per trap) and *right axes* measure overall malaria prevalence (percentage of infected individuals) in juvenile birds. Months without bars represent the months when mosquitoes were not surveyed. All abundance values refer to captures with CO_2-baited traps only

Feeding preferences

The traps baited with live animals, set in Tornada in 2009, caught the same species that were attracted to the CO_2-baited traps in the same nights (Fig. 2). Overall, 32.9 % of all individuals were attracted to the traps without any animal bait, 19.4 % to the bird-baited traps and 47.7 % to the mouse-baited traps (Fig. 2). The sample of *Cx. theileri* was too small to evaluate the species' preferences, so it was excluded from this analysis. None of the remaining five species was preferentially attracted to the bird-baited traps. The most mammophilic species was *Anopheles claviger* s.l., with 81 % of the individuals attracted to the mouse-baited trap (Kruskal–Wallis test: $H(2;24)=13.66$, $p=0.001$). Other species were also mostly attracted to the mouse bait but were not rated as significantly mammophilic in the Kruskal–Wallis test ($1.89<H(2;24)<3.90$, $p>0.14$): *An. atroparvus* (79 %), followed by *Culiseta annulata* (52 %) and *Cx. pipiens* (51 %). *Cq. richiardii* also had an opportunistic behavior ($H(2;24)=4.60$, $p=0.10$) but was more attracted to the CO_2-baited traps than to any animal bait.

Table 2 Prevalence of *Plasmodium* sp. and *Plasmodium* SGS1 in juvenile birds of the four study sites (Ventim et al. 2011)

Site	Prevalence in juvenile birds		*n*
	Plasmodium sp.	P. SGS1	(Juvenile birds)
Taipal	0.236	0.204	157
Santo André	0.228	0.198	197
Vilamoura	0.112	0.094	170
Tornada	0.204	0.194	108

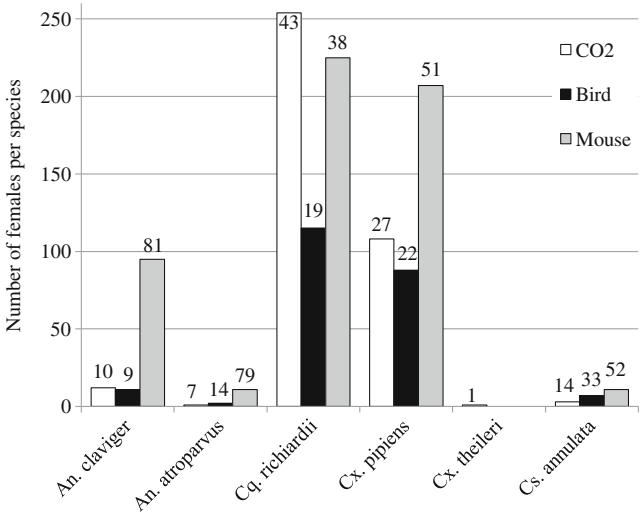

Fig. 2 Mosquito captures (number of females per species) using CO_2 (*white bars*) and two different animal baits, a bird (*L. domestica*, in *black*) or a mouse (*M. musculus*, in *gray*). *Numbers* on the bars represent the percentage of mosquitoes attracted to each of the baits. Catches took place at Tornada, on eight nights in August and September 2009

Forty engorged females were captured in Tornada (Table 3), and it was possible to identify the blood meal in seven of them. In all cases of avian donors (five cases in three mosquito species), the blood source was the Spotless Starling (*Sturnus unicolor*), a resident species that roosts in great numbers inside Tornada's reed bed.

Discussion

Mosquito abundances

Most of our results are consistent with the values found by other studies, which suggested that in Portugal, the most abundant mosquitoes are *Cx. pipiens*, *Cx. theileri*, and *Oc. caspius* (Almeida et al. 2008; Osório et al. 2010). Tornada seemed to be different because the most abundant mosquito was *Cq. richiardii*, a relatively rare species in Portugal (Almeida et al. 2008; Osório et al. 2010), and also because *Cx. theileri*, and *Oc. caspius* were practically absent, and *An. claviger* s.l. reached higher abundances than normally found in the rest of the country (Osório et al. 2010). Because Tornada was surveyed in a different year, we cannot say if its large deviation from what was previously described is an effect of environmental differences in that site or in that year. *An. atroparvus* was seldom captured in this study, despite being described as widespread and abundant in previous studies (Almeida et al. 2008); this is certainly related with our catching method, which is not the best for this species (Almeida et al. 2008).

Both the malaria prevalence in juvenile birds (Ventim et al. 2011) and the abundance of different mosquito species were significantly different between sites. In Taipal, the prevalence of P. SGS1 was higher than expected, as was the abundance of *Cx. pipiens* and *Cx. theileri*. SGS1, a lineage of the morphospecies *Plasmodium relictum* (Palinauskas et al. 2007) had already been found in species of the *Cx. pipiens* complex (Ejiri et al. 2011; Kim and Tsuda 2010; Kim et al. 2009b); *Cx. theileri* has been described as a vector of the morphospecies *P. relictum*, although no lineage was specified (Valkiūnas 2005). If these two mosquito species are implicated in the transmission cycle of SGS1, their variable distribution patterns may help to explain the different prevalences of this lineage among sites.

However, the monthly mosquito abundance was not correlated with the malaria prevalence in local juvenile passerines. This was probably because the majority of the detected bird infections belonged to the chronic phase of infection (Ventim et al. 2011; Zehtindjiev et al. 2008), which can take place several weeks after the bird has been bitten (Valkiūnas 2005).

Malaria infections

According to previous studies, the MIR for different species of mosquitoes is quite variable. For example, in different Japanese sites, the MIR for *Cx. pipiens* s.l. varies from 3.08 % (Kim and Tsuda 2010) to 0.52 % (Ejiri et al. 2009), while it was 0.03 % in the present study. Mosquito infection rates can vary in time and space and be influenced by many factors, such as the age structure of the population (Smith et al. 2004) or the infection prevalence in the hosts they feed in (Kilpatrick et al. 2006).

The fact that unengorged *Cx. pipiens* and *Cx. theileri* were infected by *Plasmodium* SGS1 and P. SYAT05, respectively, suggests that these species may be competent vectors of those lineages at the study sites. This agrees with previous studies (Ejiri et al. 2011; Kim and Tsuda 2010; Kim et al. 2009b; Valkiūnas 2005). These two lineages were also present in the passerine communities of the study sites: SGS1 was present at the four sites, in the resident Cetti's Warbler (*Cettia cetti*), and in six other resident, migrant, and exotic passerine species; SYAT05 was found in juvenile Cetti's Warblers in three of the

Table 3 Analysis of blood meals of engorged females—number of identified meals and identity of the blood donors

Mosquito species	Identified meals	Blood meal sources
Anopheles claviger s.l.	1	*Sturnus unicolor* + *Ovis aries* (1)
Anopheles atroparvus	1	*Canis lupus familiaris* (1)
Coquilletidia richiardii	2	*Sturnus unicolor* (1)
		Homo sapiens (2)
Culex pipiens	3	*Sturnus unicolor* (3)

four sites (Ventim et al. 2012). Both lineages must be transmitted locally, given that they were both present in juvenile and resident birds, which have spent most of their time in the reed bed.

In our study sites, SGS1 was the most prevalent lineage in the studied bird communities (17 % overall) (Ventim et al. 2012), and it was now found in one of the most abundant mosquito species of our study areas, *Cx. pipiens*. Therefore, we detected associations between the most common passerine species of these communities, the most abundant mosquito species, and the most common, bird-generalist *Plasmodium* lineage. Probably, the abundance of both the avian and the dipteran hosts is important to keep a good reservoir of infections in all the stages of a parasite's life cycle. SYAT05 was detected (for the first time) in another very abundant mosquito, *Cx. theileri*. This lineage is known to be host-generalist and was previously detected in many bird species (Dimitrov et al. 2010; Hellgren et al. 2007; Martinsen et al. 2007). By the former rationale, it would be expected to reach a high prevalence in the local bird hosts. However, Ventim et al. (2012) only detected an overall prevalence of 0.22 % in the passerine community of the study sites. Nevertheless, SYAT05 might infect and achieve high prevalence in unsampled local bird species, such as the Spotless Starling. Indeed, SYAT05 was detected in another population of Spotless Starlings near Madrid, Spain (Jaime Muriel, personal communication).

Feeding preferences

All the species captured with the animal baits seemed to be more attracted to the mouse than to the bird and all approximately in the same proportion. While *Anopheles maculipennis* s.l. (the species group that includes *An. atroparvus*) was described as mammophilic (Balenghien et al. 2006; Ponçon et al. 2007) and *Cq. richiardii* as an opportunistic feeder (Balenghien et al. 2006), *Cx. pipiens* is usually referred to as primarily ornithophilic (Apperson et al. 2004; Savage et al. 2007), unlike what was found in this study. Indeed, host feeding behavior may vary widely in time (Kilpatrick et al. 2006) and from location to location due to host availability (Savage et al. 2007). More importantly, mosquitoes that feed upon avian or mammal hosts target a limited number of species most of the time (Apperson et al. 2004; Kilpatrick et al. 2006; Savage et al. 2007). Thus, the exotic bird species used as bait, unknown to the local mosquitoes, was probably not a preferential target. Also, if there was a preferential bird or mammal species nearby, any other used bait would be less attractive to mosquitoes, drawing only generalist feeders or occasional individuals passing by. This may explain why *Cx. pipiens* did not appear to be ornitophilic with the bait that was used. In this case, the stronger attraction of all mosquito species to the mouse may partially be attributed to the fact that the mouse was active during the night (when the surveys took place) while the bird was asleep, so the mouse should have a higher metabolic rate and thus emit more olfactory stimulus to the mosquitoes.

In the Tornada site, the identification of blood meals made of Spotless Starling suggests that this species could be a preferential target for ornitophilic mosquitoes. The starlings are very numerous in this site and all roost together in vast dormitories, thus constituting a large source of olfactive stimulus that can a have a larger attractive power for mosquitoes than isolated birds. If they are preferential targets for *Plasmodium* vectors and they are susceptible to the locally transmitted *Plasmodium* lineages (Jaime Muriel, personal communication.), they may play an important role in the local avian malaria transmission cycles, as infection reservoirs. However, more studies would be needed to know which lineages can affect this host and which prevalence they can reach in the local host's population.

In conclusion, this study found high abundances of *Cx. pipiens*, *Cx. theileri*, *Oc. caspius* and, to a lesser extent, *Cq. richiardii*. Two *Plasmodium* lineages (SGS1 and SYAT05) were found in unengorged *Cx. pipiens* and *Cx. theileri*, respectively, suggesting that these abundant mosquitoes might be involved in the transmission cycles of those lineages. The differences in distribution patterns of these mosquitoes among sites may contribute to explain differences in parasite prevalence, as vector abundance is probably important to keep a local infection reservoir. In one of the study sites, mosquitoes seem to be attracted to the Spotless Starling, an abundant bird species that may be an important local reservoir of avian malaria infections. To our knowledge, this is the first report of detection of avian *Plasmodium* DNA from European mosquitoes.

Acknowledgments This work was financed by the Portuguese Fundação para a Ciência e Tecnologia (grant SFRH/BD/28930/2006 to R.V.). The Instituto para a Conservação da Natureza e Biodiversidade supplied permits for mosquito capture and help in the field work. The Pato Association also presented logistic support in Tornada. The authors would like to thank the contribution of Diego Gonzálvez, Helder Cardoso, Joana Morais, and Saúl Manzano to the field work. In the lab, Joana Morais, Sara Pardal and Sandra Reis were of precious help.

References

Almeida APG, Galão RP, Sousa CA, Novo MT, Parreira R, Pinto J, Piedade J, Esteves A (2008) Potential mosquito vectors of arboviruses in Portugal: species, distribution, abundance and West Nile infection. Trans Roy Soc Trop Med Hyg 102(8):823–832. doi:10.1016/j.trstmh.2008.03.011

Apperson CS, Hassan HK, Harrison BA, Savage HM, Aspen SE, Farajollahi A, Crans W, Daniels TJ, Falco RC, Benedict M, Anderson M, Mcmillen L, Unnasch TR (2004) Host feeding patterns of established and potential mosquito vectors of West

Nile virus in the eastern United States. Vector Borne Zoonotic Dis 4(1):71–82. doi:10.1089/153036604773083013

Atkinson CT, van Riper C III (1991) Pathogenicity and epizootiology of avian hematozoa: *Plasmodium*, *Leucocytozoon* and *Haemoproteus*. In: Loye J, Zuk M (eds) Bird-parasite interactions: ecology, evolution, and behavior. Oxford University Press, New York, pp 19–48

Balenghien T, Fouque F, Sabatier P, Bicout DJ (2006) Horse-, bird-, and human-seeking behavior and seasonal abundance of mosquitoes in a West Nile virus focus of southern France. J Med Entomol 43(5):936–946. doi:10.1603/0022-2585(2006)43[936:hbahba]2.0.co;2

Baylis M, ElHasnaoui H, Bouayoune H, Touti J, Mellor PS (1997) The spatial and seasonal distribution of African horse sickness and its potential *Culicoides* vectors in Morocco. Med Vet Entomol 11 (3):203–212. doi:10.1111/j.1365-2915.1997.tb00397.x

Bensch S, Stjernman M, Hasselquist D, Ostman O, Hansson B, Westerdahl H, Pinheiro RT (2000) Host specificity in avian blood parasites: a study of *Plasmodium* and *Haemoproteus* mitochondrial DNA amplified from birds. Proc R Soc B 267(1452):1583–1589. doi:10.1098/rspb.2000.1181

Bensch S, Hellgren O, Pérez-Tris J (2009) MalAvi: a public database of malaria parasites and related haemosporidians in avian hosts based on mitochondrial cytochrome b lineages. Mol Ecol Resour 9(5):1353–1358. doi:10.1111/j.1755-0998.2009.02692.x

Cicero C, Johnson NK (2001) Higher-level phylogeny of new world vireos (Aves: Vireonidae) based on sequences of multiple mitochondrial DNA genes. Mol Phylogenet Evol 20(1):27–40. doi:10.1006/mpev.2001.0944

Cox FEG (1993) Modern parasitology. Blackwell Science, Oxford

Dimitrov D, Zehtindjiev P, Bensch S (2010) Genetic diversity of avian blood parasites in SE Europe: cytochrome b lineages of the genera *Plasmodium* and *Haemoproteus* (Haemosporida) from Bulgaria. Acta Parasitol 55(3):201–209. doi:10.2478/s11686-010-0029-z

Ejiri H, Sato Y, Sawai R, Sasaki E, Matsumoto R, Ueda M, Higa Y, Tsuda Y, Omori S, Murata K, Yukawa M (2009) Prevalence of avian malaria parasite in mosquitoes collected at a zoological garden in Japan. Parasitol Res 105(3):629–633. doi:10.1007/s00436-009-1434-9

Ejiri H, Sato Y, Kim KS, Tsuda Y, Murata K, Saito K, Watanabe Y, Shimura Y, Yukawa M (2011) Blood meal identification and prevalence of avian malaria parasite in mosquitoes collected at Kushiro Wetland, a subarctic zone of Japan. J Med Entomol 48 (4):904–908. doi:10.1603/me11053

Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotechnol 3(5):294–299

Gager AB, Loaiza JDR, Dearborn DC, Bermingham E (2008) Do mosquitoes filter the access of *Plasmodium* cytochrome b lineages to an avian host? Mol Ecol 17:2552–2561. doi:10.1111/j.1365-294X.2008.03764.x

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser 41:95–98

Hellgren O, Krizanauskiene A, Valkiunas G, Bensch S (2007) Diversity and phylogeny of mitochondrial cytochrome B lineages from six morphospecies of avian *Haemoproteus* (Haemosporida: Haemoproteidae). J Parasitol 93:889–896. doi:10.1645/GE-1051R1.1

Kilpatrick AM, Kramer LD, Jones MJ, Marra PP, Daszak P (2006) West Nile virus epidemics in North America are driven by shifts in mosquito feeding behavior. PLoS Biol 4(4):606–610. doi:10.1371/journal.pbio.0040082

Kim KS, Tsuda Y (2010) Seasonal changes in the feeding pattern of *Culex pipiens pallens* govern the transmission dynamics of multiple lineages of avian malaria parasites in Japanese wild bird community. Mol Ecol 19(24):5545–5554. doi:10.1111/j.1365-294X.2010.04897.x

Kim KS, Tsuda Y, Sasaki T, Kobayashi M, Hirota Y (2009a) Mosquito blood-meal analysis for avian malaria study in wild bird communities: laboratory verification and application to *Culex sasai* (Diptera: Culicidae) collected in Tokyo, Japan. Parasitol Res 105 (5):1351–1357. doi:10.1007/s00436-009-1568-9

Kim KS, Tsuda Y, Yamada A (2009b) Bloodmeal identification and detection of avian malaria parasite from mosquitoes (Diptera: Culicidae) Inhabiting coastal areas of Tokyo Bay, Japan. J Med Entomol 46(5):1230–1234

Kimura M, Darbro JM, Harrington LC (2010) Avian malaria parasites share congeneric mosquito vectors. J Parasitol 96(1):144–151. doi:10.1645/ge-2060.1

Lanciotti RS, Kerst AJ, Nasci RS, Godsey MS, Mitchell CJ, Savage HM, Komar N, Panella NA, Allen BC, Volpe KE, Davis BS, Roehrig JT (2000) Rapid detection of West Nile virus from human clinical specimens, field-collected mosquitoes, and avian samples by a TaqMan reverse transcriptase-PCR assay. J Clinic Microbiol 38(11):4066–4071

Martinsen ES, Waite JL, Schall JJ (2007) Morphologically defined subgenera of *Plasmodium* from avian hosts: test of monophyly by phylogenetic analysis of two mitochondrial genes. Parasitology 134:483–490. doi:10.1017/s0031182006001922

Ngo KA, Kramer LD (2003) Identification of mosquito bloodmeals using polymerase chain reaction (PCR) with order-specific primers. J Med Entomol 40(2):215–222. doi:10.1603/0022-2585-40.2.215

Njabo KY, Cornel AJ, Sehgal RN, Loiseau C, Buermann W, Harrigan RJ, Pollinger J, Valkiūnas G, Smith TB (2009) *Coquillettidia* (Culicidae, Diptera) mosquitoes are natural vectors of avian malaria in Africa. Malar J 8:193. doi:10.1186/1475-2875-8-193

Njabo KY, Cornel AJ, Bonneaud C, Toffelmier E, Sehgal RNM, Valkiūnas G, Russell AF, Smith TB (2011) Nonspecific patterns of vector, host and avian malaria parasite associations in a central African rainforest. Mol Ecol 20(5):1049–1061. doi:10.1111/j.1365-294X.2010.04904.x

Osório HC, Amaro F, Zé-Zé L, Pardal S, Mendes L, Ventim R, Ramos JA, Nunes S, Workgroup R, Alves MJ (2010) Mosquito species distribution in mainland Portugal 2005-2008. Eur Mosq Bull 28

Palinauskas V, Kosarev V, Shapoval A, Bensch S, Valkiūnas G (2007) Comparison of mitochondrial cytochrome b lineages and morphospecies of two avian malaria parasites of the subgenera *Haemamoeba* and *Giovannolaia* (Haemosporida: Plasmodiidae). Zootaxa 1626:39–50

Ponçon N, Toty C, L'Ambert G, Le Goff G, Brengues C, Schaffner F, Fontenille D (2007) Biology and dynamics of potential malaria vectors in Southern France. Malar J 6. doi:10.1186/1475-2875-6-18

Ribeiro H, Ramos HC (1999) Identification keys of the mosquitoes of Continental Portugal, Açores and Madeira. Eur Mosq Bull 3:1–11

Savage HM, Aggarwal D, Apperson CS, Katholi CR, Gordon E, Hassan HK, Anderson M, Charnetzky D, Millen LMC, Unnasch EA, Unnasch TR (2007) Host Choice and West Nile virus infection rates in blood-fed mosquitoes, including members of the *Culex pipiens* complex, from Memphis and Shelby County, Tennessee, 2002–2003. Vector Borne Zoonotic Dis 7(3):365–386. doi:10.1089/vbz.2006.0602

Schaffner F, Angel G, Geoffroy B, Hervy JP, Rhaiem A, Brunhes J (2001) The mosquitoes of Europe: an identification and training programme. IRD Éditions (CD-ROM), Montepellier, France

Shurulinkov P, Ilieva M (2009) Spatial and temporal differences in the blood parasite fauna of passerine birds during the spring migration in Bulgaria. Parasitol Res 104(6):1453–1458. doi:10.1007/s00436-009-1349-5

Smith DL, Dushoff J, McKenzie FE (2004) The risk of a mosquito-borne infection in a heterogeneous environment. PLoS Biol 2(11): e368. doi:10.1371/journal.pbio.0020368

Sol D, Jovani R, Torres J (2000) Geographical variation in blood parasites in feral pigeons: the role of vectors. Ecography 23(3):307–314

Statsoft Inc (2011) Electronic statistics textbook. StatSoft. WEB: http://www.statsoft.com/textbook/, Tulsa, OK, USA

Suom C, Ginsberg HS, Bernick A, Klein C, Buckley PA, Salvatore C, LeBrun RA (2010) Host-seeking activity and avian host preferences of mosquitoes associated with West Nile virus transmission in the northeastern USA. J Vector Ecol 35(1):69–74

Valkiūnas G (2005) Avian malaria parasites and other haemosporidia, 1st edn. CRC Press, Boca Raton

van Riper C III, van Riper SG, Goff ML, Laird M (1986) The epizootiology and ecology significance of malaria in Hawaiian land birds. Ecol Monogr 56:327–344

Ventim R, Tenreiro P, Grade N, Encarnação P, Araújo M, Mendes L, Pérez-Tris J, Ramos J (2011) Characterization of haemosporidian infections in warblers and sparrows at south-western European reed beds. J Ornithol:1–8. doi:10.1007/s10336-011-0767-1

Ventim R, Morais J, Pardal S, Mendes L, Ramos JA, Pérez-Tris J (2012) Host-parasite associations and host-specificity in haemoparasites of reed bed passerines. Parasitology 139:310–316. doi:10.1017/S0031182011002083

Waldenström J, Bensch S, Kiboi S, Hasselquist D, Ottosson U (2002) Cross-species infection of blood parasites between resident and migratory songbirds in Africa. Mol Ecol 11(8):1545–1554. doi:10.1046/j.1365-294X.2002.01523.x

Waldenström J, Bensch S, Hasselquist D, Ostman O (2004) A new nested polymerase chain reaction method very efficient in detecting *Plasmodium* and *Haemoproteus* infections from avian blood. J Parasitol 90(1):191–194. doi:10.1645/GE-3221RN

White BJ, Andrew DR, Mans NZ, Ohajuruka OA, Garvin MC (2006) West Nile virus in mosquitoes of northern Ohio, 2003. AmJTrop Med Hyg 75(2):346–349

Whiteman NK, Sanchez P, Merkel J, Klompen H, Parker PG (2006) Cryptic host specificity of an avian skin mite (Epidermoptidae) vectored by louseflies (Hippoboscidae) associated with two endemic Galapagos bird species. J Parasitol 92(6):1218–1228. doi:10.1645/ge-918r.1

Zehtindjiev P, Ilieva M, Westerdahl H, Hansson B, Valkiūnas G, Bensch S (2008) Dynamics of parasitemia of malaria parasites in a naturally and experimentally infected migratory songbird, the great reed warbler *Acrocephalus arundinaceus*. Exp Parasitol 119 (1):99–110. doi:10.1016/j.exppara.2007.12.018