Rita Ventim

Haemosporidian parasites in communities of southwestern European reedbeds: their passerine hosts and their vectors

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Contents

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SUMMARY / RESUMO

Lagoa de Santo André.
Photo by: Rita Ventim (2010).

Summary

Haemosporidian parasites of the genera *Haemoproteus* and *Plasmodium* infect birds of several different families all over the world, using dipteran insects as vectors. However, the factors affecting their transmission patterns are not well known. This study examined haemoparasite infections in communities of south-western European reed beds. It characterized infection prevalence and intensity in several passerine species and investigated haemoparasite specificity, the structure of the host-parasite interactions and the geographical configuration of parasite assemblages. The distribution of local mosquito species, potential vectors of *Plasmodium* spp., was analysed and their feeding preferences were determined. Community studies involving several bird and mosquito species are important to understand the transmission patterns of haemoparasites. To my knowledge, this is the first study of this kind in Europe.

Four Portuguese red beds (Montemor-o-Velho, Tornada, Santo André and Vilamoura) were studied, including a community of 13 passerine species: six migratory warblers (four breeding, one wintering and one passing migrant), one resident warbler, two resident sparrows, two exotic finches and two exotic weavers. From 2007 to 2009, 1353 birds and over 3700 female mosquitoes from 10 species were sampled. These samples were diagnosed for haemoparasite infections using molecular techniques designed to detect the presence of haemosporidian DNA. Microscopy was used to calculate infection intensity in autochthonous birds.

Patterns of infection prevalence and intensity were characterized in resident and migratory bird species, in 2007 and 2008. 34.5% of the sampled birds were infected, all with low level parasitemias compatible with the chronic stage of infection. At the genus level, *Plasmodium* spp. infected more species and reached a higher overall prevalence. Prevalence varied between bird species and was affected by different factors in each host species, according to the host's biology. Age was important to explain prevalence in the Reed Warbler *Acrocephalus scirpaceus*, reflecting that the sampled adults had already migrated to Africa and contacted with two different parasite faunas, accumulating infections from their wintering and breeding grounds; whereas yearlings had only contacted with the local parasite community. Prevalence varied significantly with season for the resident Cetti's Warbler *Cettia cetti*, which was more infected during autumn and winter. A lower food availability in the reed beds during those seasons may weaken these birds and make them more prone to infection. This tendency was more subtle (not statistically significant) in the resident House Sparrow *Passer domesticus*, a more generalist bird that spends less time in the reed bed and can feed on other food sources; prevalence in this species only showed yearly differences.

The host-specificity of the parasite lineages was analyzed and compared with other cases described in the literature. Of the nine lineages of *Haemoproteus* and 15 of *Plasmodium* that were found, only ten *Plasmodium* lineages had confirmed local transmission. Each lineage showed a specific host preference. Host-specialist lineages did not always share hosts with generalist lineages, forming a non-nested pattern of interactions. *Plasmodium* SGS1 was the most prevalent lineage in the sampled species and also the most host-generalist. In general, the lineages with a wider host range were also the most prevalent, confirming that the ability to infect more host species increases a parasite's prevalence in its entire host range. One lineage (*H.* MW1) appeared to be significantly more host-generalist in the study sites than in previous studies from the literature. This suggests that caution is needed when accessing a parasite's specialization, since it can depend on the type of host species that are sampled.

To see if haemoparasite assemblages are geographically structured along the two main European flyways, the parasites of Reed Warblers and Great Reed Warblers *Acrocephalus arundinaceus* found in the four study sites were compared with those found in four eastern-European sites. The four Portuguese sites represented the western European flyway, while the eastern flyway was represented by four sites in Sweden, Bulgaria, Romania and Russia, taken from the literature. 32 lineages were documented for the two host species in Europe, 14 of which exclusively found in the eastern flyway, 3 exclusively found in western sites and the remaining 15 occurring along both flyways. When sites were compared two by two, nor the distance between sites nor the flyway the sites belonged to was not enough to characterize the similarity between the site's parasite assemblages. In these host species, haemoparasite assemblages show little geographical structure throughout Europe, so the migratory movements of these hosts are not enough to homogenize their parasite assemblages along their flyways. Perhaps host-generalist lineages, dispersing through the combined movements of several host species, cause this lack of structure. For this reason, local adaptation to haemoparasites seems to constitute a poor selective agent in the evolution and maintenance of birds' migratory connectivity.

To test if the exotic passerine species had benefitted from a release from haemoparasites, their infections were compared with those found in six local bird species. The exotic birds had less infecting lineages and a lower overall prevalence, but this difference was not significant once phylogeny was controlled for. Two local *Plasmodium* lineages infected the exotic species: the generalist *P.* SGS1 was the most prevalent lineage in the native species, so it could be expected to be present in the exotics at random. *P.* PADOM01 was rarer in the sampled community, but was present in native sparrows (phylogenetically close to the infected exotic species), so its colonization of the exotic host must be aided by its specialization in Passeroids. Therefore, the enemy release hypothesis did not seem to apply to the haemoparasites

of these exotic species, as they were infected by local haemoparasites in the same way as the local host species.

The spatial distribution of local vectors of *Plasmodium* sp. was investigated, together with their relations both with parasites and with birds (through the mosquito's feeding preferences). In three of the study sites, the most abundant mosquito species were *Culex pipiens*, *Cx. theileri* and *Ochlerotatus caspius*. In the Tornada site, *Coquilletidia richiardii* was very abundant, but *Cx. theileri* and *Oc. caspius* were absent. Unengorged *Cx. pipiens* and *Cx. theileri* were infected by two locally transmitted lineages, *P.* SGS1 and *P.* SYAT05 (respectively), suggesting those mosquitoes as competent vectors of these lineages. These species' abundance was significantly different among sites, which may help to explain the observed differences in the prevalence of *P.* SGS1. This lineage was detected in the most abundant mosquito species and reached a high prevalence in the most abundant passerine species; possibly, this parasite needs abundant hosts in all phases of its cycle to keep a good reservoir of infection in all its stages. In an experiment using CO_2 and animal baited traps at Tornada, *Cq. richiardii* appeared to be an opportunistic feeder. *Cx. pipiens* appeared to be mammophilic (contrarily to previous descriptions), perhaps because the used avian bait was unknown to the local mosquitoes. The identification of blood meal donors points to the Spotless Starling *Sturnus unicolor* as a possible preferential target in that site.

Resumo

Parasitas hemosporídeos dos géneros *Haemoproteus* e *Plasmodium* infectam aves pertencentes a diversas famílias em todo o mundo, usando insectos dípteros como vectores. No entanto, ainda não se conhecem bem os factores que afectam os seus padrões de transmissão. Este estudo examinou infecções por hemoparasitas em comunidades de caniçais do sudoeste europeu. A prevalência e intensidade das infecções foram caracterizadas em várias espécies de passeriformes e investigou-se a especificidade de hospedeiro dos parasitas, a estrutura das relações hospedeiro-parasita e a configuração geográfica das comunidades de parasitas. Analisou-se a distribuição das espécies de mosquito locais (potenciais vectores de *Plasmodium* spp.) e as suas preferências alimentares. Estudos a nível das comunidades, envolvendo várias espécies de aves e de mosquitos, são importantes para o entender os padrões de transmissão dos hemoparasitas. Tanto quanto eu sei, este é o primeiro estudo dessa natureza feito na Europa.

Investigaram-se quatro caniçais portugueses (Montemos-o-Velho, Tornada, Santo André e Vilamoura), abrangendo uma comunidade de 13 espécies de passeriformes: seis felosas migradoras (quatro nidificantes, uma invernante e uma migradora de passagem), uma felosa residente, dois pardais residentes, dois fringilídeos exóticos e dois tecelões exóticos. De 2007 a

2009, amostraram-se 1353 pássaros e mais de 3700 mosquitos fêmea. Todas estas amostras foram analisadas para diagnosticar infecções de hemoparasitas, usando técnicas de biologia molecular que detectam a presença do ADN do parasita. Recorreu-se à microscopia para calcular a intensidade das infecções em aves autóctones.

Caracterizaram-se os padrões de prevalência e intensidade de infecção em aves residentes e migratórias, em 2007 e 2008. 34,5% dos pássaros amostrados estavam infectados, todos eles com infecções de baixa intensidade compatíveis com a fase crónica. Ao nível do género do parasita, *Plasmodium* spp. infectou maior número de espécies e atingiu prevalências mais altas que *Haemoproteus* spp.. A prevalência variou entre espécies de ave e foi afectada por factores diferentes em cada espécie, segundo a biologia do hospedeiro. A idade foi importante para explicar a prevalência no rouxinol-dos-caniços *Acrocephalus scirpaceus*, reflectindo o facto de os adultos já terem migrado para África e assim terem contactado com duas faunas parasíticas diferentes, acumulando infecções dos seus locais de invernada e nidificação; enquanto os juvenis apenas tinham contactado com a comunidade local de parasitas. A prevalência variou significativamente com a estação do ano para o residente rouxinol-bravo *Cettia cetti*, que estava mais infectado durante o outono e o inverno. a menor disponibilidade de alimento nos caniçais durante essas estações pode enfraquecer estas aves, tornando-as mais susceptíveis às infecções. Esta tendência foi mais subtil (não estatisticamente significativa) no pardal *Passer domesticus*, uma ave residente mais generalista, que passa menos tempo no caniçal e pode depender de outras fontes de alimento; de facto, a prevalência no pardal só mostrou diferenças entre anos.

A especificidade de hospedeiro das várias linhagens de parasitas foi analisada e comparada com outros casos descritos na literatura. Das nove linhagens de *Haemoproteus* e 15 de *Plasmodium* que foram encontradas, só dez linhagens de *Plasmodium* é que tinham transmissão local confirmada. Cada linhagem demonstrou ter uma preferência de hospedeiro específica. As linhagens especialistas nem sempre partilhavam hospedeiros com as generalistas, formando um padrão de interacções não aninhado. O *Plasmodium* SGS1 foi a linhagem mais prevalente nas aves amostradas, e também a mais generalista. Em geral, as linhagens com um espectro de hospedeiros mais amplo foram também as que tiveram maiores prevalências, conformando que a capacidade de infectar mais espécies de hospedeiro aumenta a prevalência em todas essas espécies de hospedeiro. Houve uma linhagem (*H.* MW1) que parecia ser significativamente mais generalista nos locais de estudo do que em estudos anteriores da literatura. Isso sugere que o grau de especialização de um parasita deve ser determinado com cautela, pois pode depender do tipo de hospedeiro que é amostrado.

Verificou-se se as comunidades de hemoparasitas são estruturadas geograficamente ao longo das principais rotas migratórias dos seus hospedeiros. Para tal, os parasitas de rouxinóis-dos-caniços e rouxinóis-grandes-dos-caniços *Acrocephalus arundinaceus* encontrados nos

quatro locais de estudo foram comparados com os que foram descritos em quatro locais do leste da Europa. Os quatro sítios portugueses representaram a rota migratória do oeste europeu, enquanto a rota do leste foi representada por quatro sítios na Suécia, Bulgária, Roménia e Rússia, retirados da literatura consultada. Documentaram-se 32 linhagens para estas espécies de hospedeiro na Europa, das quais 14 exclusivamente na rota de leste, 3 exclusivamente na rota do oeste e as restantes 15 eram ocorriam em ambas as rotas. Quando os locais foram comparados dois a dois, nem a distância entre locais nem a rota à qual pertenciam foi suficiente para caracterizar a semelhança entre comunidades de parasitas. Nestes hospedeiros, as comunidades de hemoparasitas apresentam muito pouca estruturação geográfica, pelo que os movimentos migratórios destes hospedeiros não são suficientes para homogeneizar as comunidades parasíticas ao longo das suas rotas migratórias. Talvez esta falta de estrutura seja causada pelos parasitas generalistas, que se dispersam através dos movimentos combinados de várias espécies de hospedeiro. Por este motivo, a adaptação local aos hemoparasitas parece exercer uma pressão selectiva demasiado baixa para moldar a evolução e a manutenção da conectividade migratória destes pássaros.

Para testar se as aves exóticas beneficiaram de uma libertação dos seus hemoparasitas, as suas infecções foram comparadas com as infecções de seis espécies locais. As aves exóticas eram infewctadas por menos linhagens e com uma prevalência mais baixa, mas esta diferença não era significativa depois de se controlar a análise para o efeito da filogenia. Havia duas linhagens locais de *Plasmodium* a infectar as aves exóticas: o generalista *P.* SGS1 era a linhagem mais prevalente nas espécies nativas, pelo que a sua presença nas exóticas poderia ser esperada por processos aleatórios. *P.* PADOM01 era mais raro na comunidade amostrada, mas estava presente em pardais natives (filogeneticamente próximos das espécies exóticas infectadas), portanto a sua colonozação dos hospedeiros exóticos deve ter sido ajudada pela sua especialização em aves da família Passeridae. Assim, a hipótese da libertação dos inimigos não parece aplicar-se aos hemoparasitas destas espécies exóticas, pois elas foram infectadas por hemoparasitas locais do mesmo modo que os hospedeiros nativos.

A distribuição dos vectores locais de *Plasmodium* sp. foi estudada, junto com suas relações destes tanto com os parasitas, como com as aves (através das preferências alimentares dos mosquitos). Em três dos locais estudados, as espécies de mosquitos mais abundantes eram *Culex pipiens*, *Cx. theileri* e *Ochlerotatus caspius*. Na Tornada, *Coquilletidia richiardii* foi muito abundante, mas *Cx. theileri* e *Oc. caspius* estiveram ausentes. Foram encontrados *Cx. pipiens* e *Cx. theileri* não engurgitados infectados por duas linhagens de transmissão local, *P.* SGS1 e *P.* SYAT05 (respectivamente), o que sugere que estes mosquitos podem ser vectores competentes dessas linhagens. A abundância dessas espécies variou significativamente entre locais, o que pode contribuir para as diferenças observadas na prevalência de *P.* SGS1. Esta linhagem foi detectada na espécie mais abundante de mosquito e tinha alta prevalência nas

espécies mais abundantes de pássaros; possivelmente, este parasita precisa de hospedeiros abundantes em todas as fases do seu ciclo de vida, para manter um grande reservatório de infecção em todos os seus estados. Num teste às preferências alimentares envolvendo CO_2 e iscos animais na Tornada, *Cq. richiardii* pareceu ser oportunista. *Cx. pipiens* pareceu ser mamofílico (contrariamente às descrições anteriores desta espécie), talvez por a ave usada como isco ser desconhecida para os mosquitos locais. A identificação dos dadores de sangue de refeições de mosquito aponta para o estorninho-preto *Sturnus unicolor* como espécie alvo preferencial dos mosquitos na Tornada.

GENERAL INTRODUCTION

Ringing material, all set for action, in Tornada

Photo by: Rita Ventim (2009).

General Introduction

What are haemosporidians? And why do we care?

Parasitism is an intimate relationship between two different species in which one (the parasite) uses the other (the host) as its environment from which it derives nourishment, sometimes for a prolonged time (Friend and Franson 1999). Parasitism can affect the host's survival and fitness (Atkinson and van Riper III 1991) and, like predators or food availability, it can regulate the host's populations (Anderson and May 1978). Interest in the potential influence of parasite pressure in the host's ecology and evolution has grown in the past decades. In general, the spread of pathogens due to global climate changes and human-induced opening of wild areas can potentially threaten wildlife (Vitousek et al. 1997). To evaluate such a threat, it is important to improve the knowledge of host-parasite relationships, in order to understand the susceptibility of populations to new diseases.

Haemosporidians (Sporozoa: Haemosporida) are a group of endoparasitic protists that inhabit a broad range of host species of amphibians, reptiles, birds and mammals and use blood-sucking dipteran insects as vectors. These protozoans are worldwide distributed and spread through a wide range of habitats and geographical regions (Valkiūnas 2005). There are three main genera of haemosporidians that infect birds: *Plasmodium*, which is the causal agent of true avian malaria, and *Haemoproteus* and *Leucocytozoon*, which cause other related haemosporidioses. Based on morphological variation, about 175 species have been described so far within the genera *Plasmodium* and *Haemoproteus* (Valkiūnas 2005); however, recent molecular-based studies (Bensch et al. 2004; Ricklefs et al. 2004; Waldenström et al. 2004) imply that the species diversity is much larger in these genera. These parasites have been recorded in about 68 per cent of the avian species that have been examined and infect almost every order of birds, of which the richest haemosporidian fauna is found in the order Passeriformes (Valkiūnas 2005; Peirce 1981a). There are marked differences in infection rates between and within avian orders, which are likely to reflect different components such as exposure to vectors, habitat choice and migration strategy or even host behaviour, resistance or susceptibility (Valkiūnas 2005; Atkinson and van Riper III 1991).

To date, the identification of haemosporidians has been based on their morphology (observed by microscopy) and biological characteristics, such as vectors and host range (Valkiūnas 2005). However, these characters may not accurately reflect phylogenetic relationships among parasites (Escalante et al. 1998) and are not enough to distinguish between cryptic species. As a consequence, the species concept for haemosporidians is still under debate. Recently, the use of molecular techniques has revealed extensive genetic diversity which has

made it possible to better address these issues (Beadell et al. 2004; Bensch et al. 2004; Ricklefs and Fallon 2002; Waldenström et al. 2002). Using polymerase chain reactions (PCR) to find variation in the parasite's cytochrome b gene, these studies defined parasite lineages, which greatly outnumber the traditional morpho-species. This mitochondrial variation is associated with nuclear DNA variation and these lineages do not seem to recombine even when they co-occur inside the same host (Bensch et al. 2004); therefore, they may be considered as separate species (Pérez-Tris et al. 2007; Bensch et al. 2004). These molecular techniques are also more sensitive than traditional microscopy (Jarvi et al. 2002), improving the diagnosis of low intensity parasite infections.

Avian haemosporidians are responsible for many diseases in domestic birds, decreasing the host's productivity and even causing high mortality (Valkiūnas 2005). Although their effects on wild bird populations have not yet been fully understood, negative effects on fitness have already been proven (Schrader et al. 2003; Sol et al. 2003; Merino et al. 2000a). These are the drivers of a co-evolutionary process between parasite virulence and specialization, and host resistance or avoidance mechanisms (Woodworth et al. 2005; Ricklefs and Fallon 2002). In this way, haemoparasites seem to have an ecological and evolutionary role in their hosts' lifes (Stjernman et al. 2008).

The worldwide distribution and accessible avian hosts of haemosporidians make them attractive models to study the ecology, evolution and epidemiology of infectious diseases. These parasites have also served as model organisms for studies on many aspects of host-parasite interactions, including host-parasite co-evolution (Ricklefs and Fallon 2002), host life-history trade-offs (Richner et al. 1995), the role of pathogens in invasive processes (Marzal et al. 2011; Lima et al. 2010) and sexual selection (Hamilton and Zuk 1982). Avian malaria is not likely to become infective to humans, given that the parasites that cause it do not have a tendency to switch to mammal hosts (Escalante et al. 1998). Despite of this, avian malaria can be used as a model for the study of human malaria or other infectious diseases that do threaten human health.

Haemosporidian life cycles and avian haemosporidioses

Haemosporidians are heteroxenous parasites, that is, there is more than one obligatory host type in their life cycles. Their intermediate hosts (organisms that only harbour non-sexual parasite stages) are vertebrates, while their definitive hosts (the ones in which mature and sexual stages occur) are dipteran insects (Fig. 1). Because these dipterans transmit infective stages between bird hosts, they are also referred to as vectors of avian haemosporidians.

Several minutes after the vector has fed on an infected bird, the parasites escape from the avian erythrocytes into the dipteran's midgut. Gametogenesis and sexual reproduction take

place there, followed by asexual reproduction (sporogony) that originates spreading stages called sporozoites. The developed sporozoites, infective to birds, move through the vector's haemocoel into its salivary glands. Transmission to the avian host occurs while the insect feeds on the blood of an appropriate bird (Valkiūnas 2005; Atkinson and van Riper III 1991).

Inside the bird's body, the sporozoites move into the cells of fixed tissues and undergo asexual division, producing meronts or schizonts a few days after the infection date (Fig. 1). With several rounds of multiple division (merogony or schizogony), meronts originate merozoites, which are the asexual stages of distribution within the host's organism (the number of merogony rounds and the organs where it occurs depend on the parasite in question).

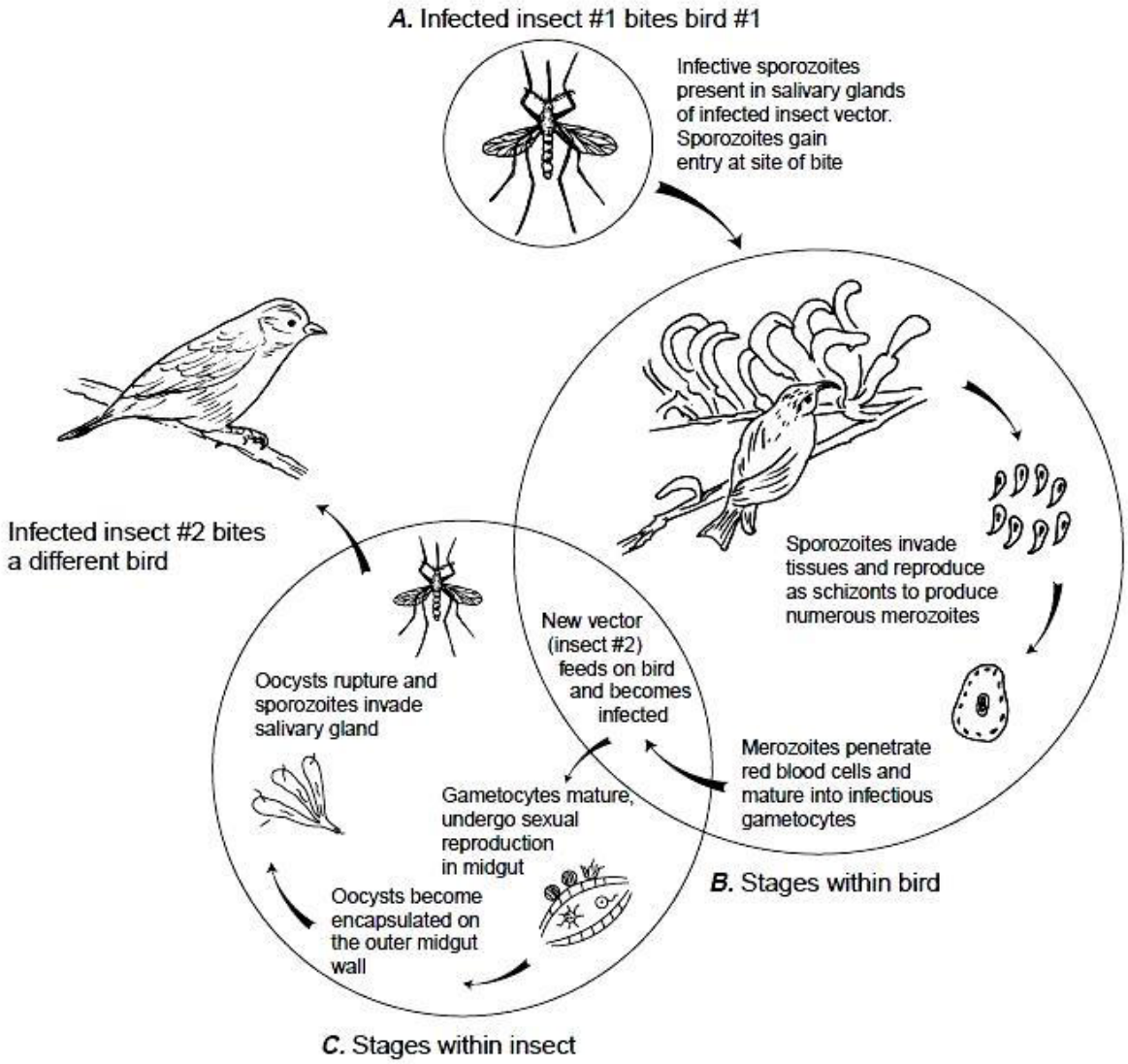


Figure 1. Illustration of the general life cycle of haemosporidian parasites.

A) Infection of a bird host through the bite of an infected insect; B) Asexual division and maturation inside the bird host; C) Sexual reproduction and sporogony inside the insect vector. (From: Atkinson 1999).

Merozoites come out of the fixed tissues, spread into the blood stream and invade the erythrocytes. There, they can continue merogony/schizogony (*Plasmodium*) or they can develop directly into gametocytes of both genders (*Haemoproteus* and *Leucocytozoon*). These gametocytes are infective to vectors (Valkiūnas 2005; Atkinson and van Riper III 1991).

Species of *Haemoproteus*, *Plasmodium* and *Leucocytozoon* are closely related genetically but differ in life-history traits. *Plasmodium* is the only genus to undergo schizogony in fixed as well as circulating blood cells, so the presence of both schizonts and gametocytes inside erythrocytes diagnoses *Plasmodium* spp. infections (Atkinson and van Riper III 1991). These are the only infections that are called avian malaria. *Leucocytozoon* spp. merozoites can invade not only erythrocytes, but also mononuclear leukocytes, inside which they also develop into gametocytes (Valkiūnas 2005).

The proportion of infected red blood cells (named parasitemia or parasite intensity) and the symptoms of haemosporidioses change in time according to different phases of the disease. The infection in birds includes the following main periods: 1 – prepatent, when the parasites first develops in an internal organ; 2 – acute, when parasites appear in the blood stream (becoming detectable in blood smears) and parasitemia increases sharply; 3 – crisis, when parasitemia reaches its peak; and 4 – chronic or latent, when immune response reduces parasitemia in circulating blood cells to low levels and surviving hosts show little or no signs of infection. In the wild, birds usually maintain chronic infections for many years or for life. Parasites may relapse in the blood during stressful situations for the hosts (Bennett et al. 1993; Atkinson and van Riper III 1991), especially during the breeding period (spring relapse), facilitating the infection of vectors (Valkiūnas 2005).

Different parasites, even from the same genus, can have quite different pathological effects. Several studies have revealed severe anaemia for parasite species of the three genera; other significant effects, such as poor thermoregulation, pneumonia, cerebral lesions or muscular cysts, are associated with particular species, both in the prepatent and in the erythrocytic (acute) phases of infection (Atkinson and van Riper III 1991). Effects are greater during the primary infection, when the naïve individual host has its first encounter with the parasite (Bensch et al. 2007; Valkiūnas 2005). Avian haemosporidioses can be severe or even lethal for domestic birds and for birds in zoos (Ferrell et al. 2007), which are not adapted, or have not co-evolved with these diseases. There are also some examples of weakening, illness and death of wild individuals caught with acute haemosporidioses; however, the effects of haemosporidioses in the wild are not well known, since most studies were conducted with domesticated or captive birds (Valkiūnas 2005).

Effects on wild host populations

At the population level, haemoparasites can affect their hosts by reducing fitness parameters such as body condition, reproductive success and survival (Norte et al. 2009; Stjernman et al. 2004; Schrader et al. 2003; Merino et al. 2000a). However, relatively few studies have been conducted with wild populations and the evidence for negative impacts on host condition is still mixed.

There are few reports of mortality caused by blood parasites in wild birds and many studies find no negative impact on individual survival rates (Stjernman et al. 2004; Bennett et al. 1993; Davidar and Morton 1993). However, mortality in the wild is very hard to monitor and severely ill individuals will rarely be caught and sampled (Valkiūnas 2005); therefore, in most studies of wild populations, most of the sampled infections are in the chronic phase and have smaller fitness costs for the host (Bensch et al. 2007). On the other hand, research in Hawaii, where malaria parasites (*Plasmodium relictum*) were only introduced last century, shows high mortality rates in the native, bird populations that did not co-evolve with these parasites (Atkinson et al. 2000; Atkinson et al. 1995; van Riper III 1991). Other investigations have found that infections with *Plasmodium* and *Haemoproteus* can affect the host's survival and health state and alter hemato-serological and immune parameters (Stjernman et al. 2008; Valkiūnas 2005; Schrader et al. 2003; Ots and Horak 1998; Richner et al. 1995).

Parasitemia and reproduction costs

It has been suggested that the cost of reproduction in birds could be mediated by a trade-off between energy allocation in reproduction and in defence against haematozoan parasites (Oppliger et al. 1996; Richner et al. 1995). Lowering the investment in immune function is at least part of the mechanism that regulates this trade-off (Martin et al. 2007; Sheldon and Verhulst 1996). According to the parasite influence hypothesis, the presence of parasites that the host's body needs to fight will reduce the energetic resources available for current reproduction (Martin et al. 2007; Møller 1997; Atkinson and van Riper III 1991). This correlation can be found by medically reducing the parasite loads of infected birds, which allows the hosts to decrease their investment in immune response and increase the reproductive effort (Tomás et al. 2007; Marzal et al. 2005; Merino et al. 2000a).

On the other hand, the more resources are invested in current reproduction, the less is available for parasite resistance, rendering the host more susceptible to new infections or relapses. Clutch size and brood size manipulation experiments show that reproduction effort can be positively correlated with subsequent parasite load (Oppliger et al. 1996; Ots and Horak 1996; Richner et al. 1995). Given that parasitemia can lower survival chances, this corresponds to an investment in current reproduction at expenses of future reproduction.

Sexual selection and parasite resistance

One of the prominent features of avian malaria is a wide variation among host species in parasite *prevalence* – that is, in the proportion of individuals infected with haematozoa in a population. Among other factors, prevalence is affected by the immunological capacity of the host to either prevent parasite infection or to clear established infections (Atkinson and van Riper III 1991).

Focusing on variation in immune system competence within populations, Hamilton and Zuk (1982) associated parasite prevalence with plumage brightness in passerine species. Costly secondary sexual traits such as plumage brightness may have evolved as indicators of "good genes", such as those implicated in parasite resistance. If parasites reduce host fitness, the Hamilton-Zuk hypothesis predicts a negative relationship between the presence and/or quantity of parasites and sexual ornaments, as only the healthiest individuals (with a better immune response) will be able to pay the costs of fully developed sexually selected traits. Some comparative between-species analyses have found a positive association between parasite prevalence and plumage brightness (Scheuerlein and Ricklefs 2004), but others have failed to find this relationship (Ricklefs et al. 2005). Within species, many studies (Votýpka et al. 2003; Hamilton and Poulin 1997) have found a significant over-all negative correlation between parasite presence and plumage showiness, supporting this hypothesis.

Habitat choice and parasite avoidance

In some cases, haemosporidioses have such severe fitness effects that they influence the host's habitat choice. Bird species that are unable to develop resistance to the parasites may have more difficulty to use areas or habitats where vector abundance increases the chances of infection. In Hawaii, the introduction of *Plasmodium relictum* and its exotic vector *Culex quinquefasciatus* was shown to reduce the distribution area of many forest birds, which could no longer colonize lower elevation habitats where the mosquito was abundant (Woodworth et al. 2005; Atkinson et al. 2000; Atkinson et al. 1995). Such constrains can also occur in long-distance migratory birds that are subject to infection in their winter quarters. This was found in wintering communities of reed bed passerines (Waldenström et al. 2002) and of shorebird species (Mendes et al. 2005), for which malaria prevalence was lower in coastal habitats than in freshwater, inland marshes.

The vectors of haemosporidioses

Malaria parasites (*Plasmodium* spp.) only develop in female mosquitoes, most frequently of the Genus *Culex*. *Haemoproteus* spp. use *Culicoides* midges and hippoboscid flies

as vectors, while *Leucocytozoon* spp. use simuliid flies (Valkiūnas 2005; Atkinson and van Riper III 1991). Every haemosporidian species can use a number of different dipteran vectors, although the specific list of vectors for most parasite species has not been determined yet.

Humid, warm, low salinity habitats are ideal for vector growth, as both mosquitoes and biting midges develop their eggs and larvae on stagnant fresh water surfaces (Cox 1993). However, even close-by locations can have rather different vector communities; the environmental characteristics of each habitat influence vector abundance, which affects transmission and prevalence of parasites (Sol et al. 2000). Climatic variation affecting vector populations has been proposed as explanation for cyclic variations in malaria prevalence, which have been observed in humans, lizards (Schall and Marghoob 1995) and one warbler species (Bensch et al. 2007).

Transmission rate of haemosporidians is a function of the abundance, hosts specificity and ecological requirements of their vectors (van Riper III et al. 1986). Even colonization of new areas by the parasites depends, among other factors, on the existence of appropriate vectors in those areas. To fully understand parasite evolution and transmission, knowledge of all three components of the vector-host-parasite system is essential. But little is known about parasite-vector associations in the wild, although predictions are that most parasites should be vector generalists, not tightly coevolved with determined vector species (Njabo et al. 2011; Kimura et al. 2010).

Transmission in passerine communities

Haemosporidian transmission to avian hosts may occur either in discrete seasons or throughout the year, depending on climate, region, parasite life cycle and vector distribution (Waldenström et al. 2002; Atkinson and van Riper III 1991). Some haemosporidians can switch hosts between resident and migrant populations of a single bird species (Pérez-Tris and Bensch 2005b) or between different species, sometimes even from different families (Hellgren et al. 2009; Waldenström et al. 2002; Bensch et al. 2000). Phylogenies based on cytochrome b sequences suggest that cross-species transmission is common among songbirds and that host shifts have occurred repeatedly during the evolution of the host-parasite system (Ricklefs et al. 2004; Waldenström et al. 2002; Bensch et al. 2000). If these host-generalist parasites are capable of year-round transmission, they can infect migrant hosts and then use the bird's migration to colonize distant areas, where they can find new vectors and infect new hosts (Waldenström et al. 2002; Pérez-Tris and Bensch 2005b). Parasite dispersal from one biographical zone to another seems to be a rare and slow evolutionary process that leaves

phylogenetic markings in the genus *Haemoproteus*, but it seems to occur more frequently in the genus *Plasmodium* (Hellgren et al. 2007b).

Reed bed passerines are known to suffer from relatively high rates of haemosporidian infection (Valkiūnas 2005; Waldenström et al. 2002; Bensch et al. 2000). The majority of European reed bed passerines are long-distance migrants that spend the winter in African freshwater marshes, where the likelihood of infection with parasites is high (Waldenström et al. 2002). On their journey, these migrant birds encounter different faunas of parasites in separated geographical areas, thus can acquire different infections. Tropical blood parasites may use resident African birds as reservoirs throughout the year and then infect the migrant birds as soon as they reach their African wintering quarters (Waldenström et al. 2002). In spring, these migrants return to Europe, where they stop to refuel or settle for breeding in the many European reed bed areas.

The importance of the Portuguese marshes

Portuguese wetlands, such as freshwater marshes, harbour a variety of ducks, waders, passerines and other waterbirds, both migratory and resident (Neto 2003). Being some of the most south-western points of Europe, these areas are among the last stop-over sites for migrant birds heading to Africa, before they cross the Mediterranean Sea and the Sahara desert. Their reed beds (areas of vegetation dominated by common reed, *Phragmites australis*) sustain bird communities that change seasonally in response to the reed's growth patterns. In winter and early spring, when the reed's productivity is lower, there are fewer birds and mostly resident species like the Cetti's Warbler *Cettia cetti* can be found. During the reproductive season, these areas are important breeding sites for residents and for migrants such as the Reed and the Great Reed Warblers, *Acrocephalus scirpaceus* and *A. arundinaceus*. During autumn migration, the numbers and the diversity of passerines greatly increase, as species of passing migrants like the Willow Warbler *Phyllosocpus trochilus* stop to refuel in great numbers (Neto 2003). The still waters of these sites are also good breeding areas for several species of mosquitoes, being the most common species *Ochlerotatus caspius*, *Culex pipiens* and *Culex theileri* (Osório et al. 2010). This creates a strong potential for the transmission of avian malaria. Despite the importance of these areas in sustaining migratory species and local biodiversity, previous knowledge of haemosporidian infections in south-western European reed beds is very scarce.

The present work took place in the reed beds of four Portuguese wetlands (Fig. 2): Paul do Taipal, in Montemor-o-Velho (N 40º11', W 8º41'); Paul de Tornada, in Tornada (N 39°26', W 9°08'); Santo André Lagoon, near Aldeia de Brescos (N 38°04', W 8°48'); and Vilamoura Environmental Park, in Vilamoura (N 37º04', W 8º07'). All of these areas are

located at sea level and close to the coast (from 1.5 to 16 Km away from the ocean). Santo André is a brackish water coastal lagoon, while the other three are freshwater marshes. The importance of these areas for the local bird fauna is shown by the fact that Tornada and Santo André are classified as nature reserves and Ramsar sites, while Taipal is an Important Bird Area and also a Ramsar site. The Vilamoura Envorinmental Park is included in the Agricultural and Ecological National Reserve.

These wetlands are aligned along a latitude gradient of 370 Km. These latitude differences are associated with a gradient of the average annual temperatures, which tend to ride from the north to the south (from 15.9ºC in Taipal to 17.7ºC in Vilamoura). Such differences in the local weather conditions, together with differences in the vegetation or surrounding areas, can originate differences in the community of vectors, parasites and hosts or in the type of interactions between them.

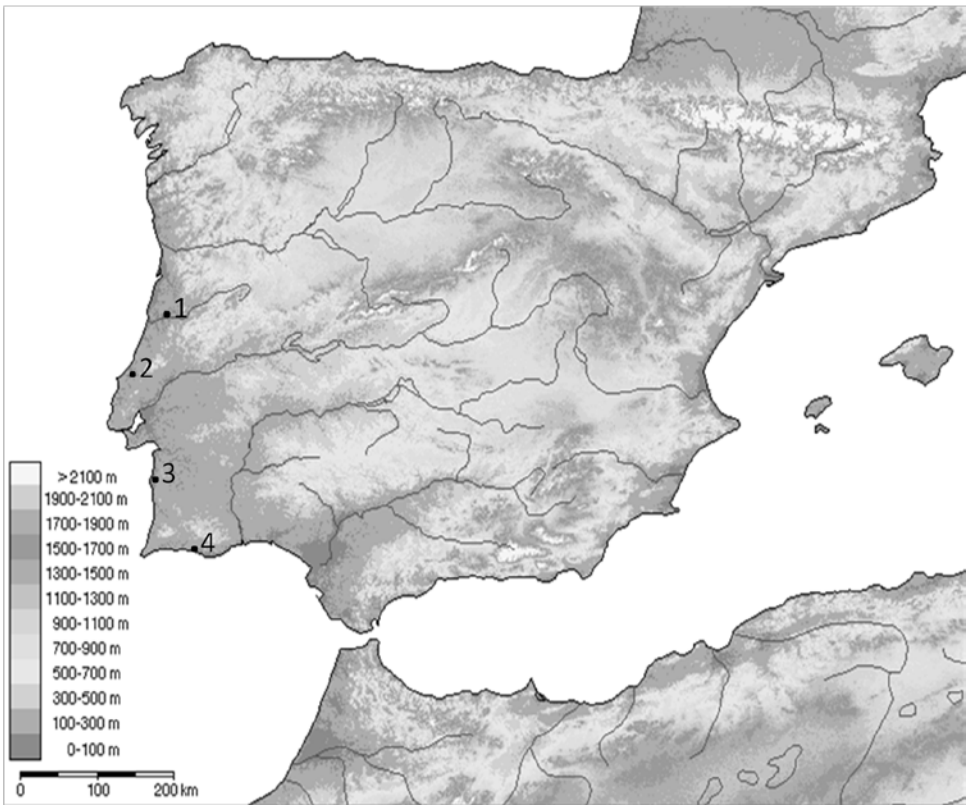


Figure 2. The Iberian Peninsula with the location of the study areas, in four Portuguese wetlands: 1-Paul do Taipal, 2-Paul de Tornada, 3-Lagoa de Santo André, 4-Caniçal de Vilamoura.

Dissertation outline

This work studied the ecology and transmission patterns of avian haemosporidians (genera *Haemoproteus* and *Plasmodium*) in a community of reed bed passerines, including migratory and resident species, at four Portuguese wetlands. The general objective was to characterize the interactions between haemoparasites, their avian hosts and their vectors in this ecosystem. The particular questions that were raised were: which factors influence the prevalence and intensity of haemosporidian infections (chapter 1); what is the host specificity of haemosporidians and the structure of the host-parasite interactions (chapter 2); can haemoparasites be used to study migratory connectivity of migrant populations (chapter 3); did they affect the colonization process of exotic bird species (chapter 4); what dipteran species are available as vectors in the studied areas and which malaria lineages can they transmit to passerines (chapter 5).

Overall, these issues were addressed by sampling 1353 birds from 13 passerine species and over 3700 female mosquitoes from 10 species, in four coastal Portuguese reed beds, from 2007 to 2009. All these samples were diagnosed for haemoparasite infections using modern molecular techniques, designed to detect the presence of parasite's DNA. Sampling such a large number of passerine and mosquito species was innovative and provided a better understanding of the general ecology of this complex system of host-parasite-vector interactions.

Chapter one provides a general description of the infection prevalence and intensity in the autochthonous hosts, combining molecular detection methods with microscopy to diagnose infections. This analysis focuses on the avian hosts, differentiating the haemoparasites only to the genus level and measuring the effect of several variables on the probability of infection of the host. It reveals that infections in different host species are affected by distinct variables, according to the host's biology.

Chapter two focuses on the parasites that occur in the study sites, using molecular methods to discriminate between different lineages. It establishes which lineages are transmitted in the study areas and what their host specificity is, suggesting that a parasite's overall prevalence and host range are connected. It also establishes a non-nested pattern of host-parasite interactions and explores the ecological consequences of such a pattern for the parasites.

In chapter three, the geographical structure of haemoparasite assemblages across Europe was investigated in two migratory host species. The parasite communities in those hosts at the four study sites (located in the western European flyway) were compared with the parasite communities of sites located in the eastern flyway (compiled from bibliography). The parasite community is considered to have little geographical structure throughout Europe.

Chapter four deals with haemosporidian infections in exotic bird populations, establishing that these introduced bird species are susceptible to some local parasites. This was

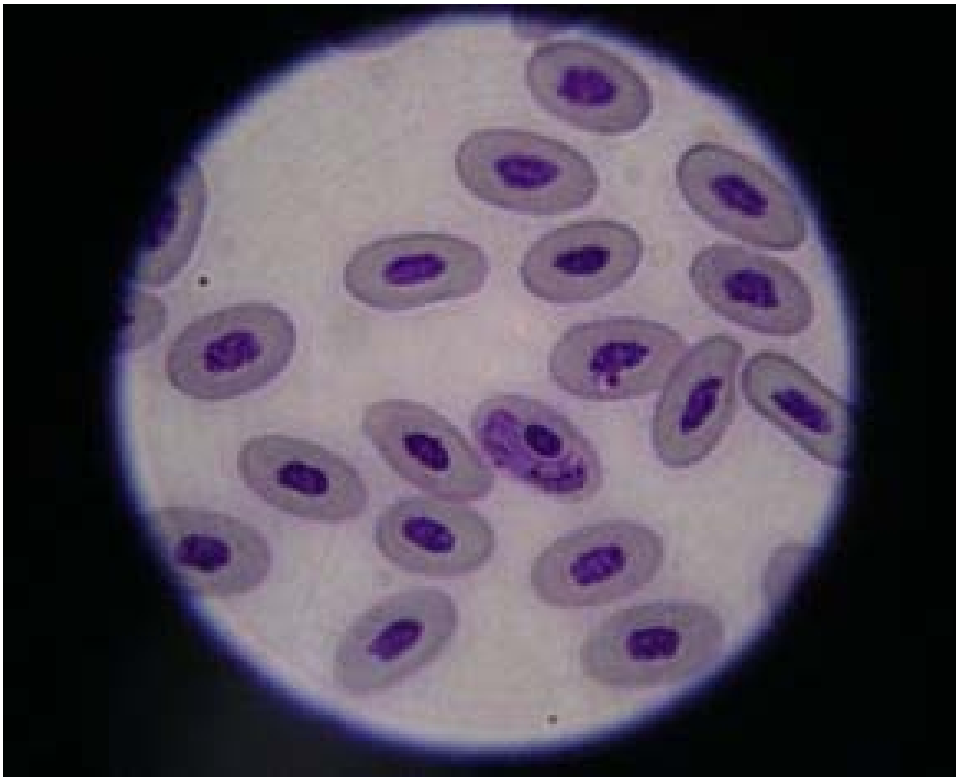
the first time that the host-parasite associations were studied for these exotic populations and that the role of haemoparasites in their colonization success was analysed.

Chapter five analyses mosquito communities, identifying possible vector species present in the study sites and their diagnosed malaria infections. Taken together with the data on bird infections (reported in the previous chapters) and on the identity of blood donors of mosquito meals, these results allow the identification of some vector-parasite associations and host-vector interactions, providing a more complete knowledge of this complex system of species interactions.

CHAPTER 1

Characterization of haemosporidian infections in warblers and sparrows at south-western European reed beds

Rita Ventim, Paulo Tenreiro, Nuno Grade, Paulo Encarnação, Miguel Araújo, Luísa Mendes, Javier Pérez-Tris and Jaime A. Ramos



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Haemoproteus spp. inside an erythrocyte in an avian blood smear.
Photo by: Ibone Anza (CERVAS, 2009)

Characterization of haemosporidian infections in warblers and sparrows at south-western European reed beds

Abstract

The prevalence and intensity of avian haemosporian infections (genera *Haemoproteus* and *Plasmodium*) was investigated using molecular techniques and microscopy in nine passerine species at three Portuguese reed beds along a small latitudinal gradient. The effect of age, sex, season, site and year in the infection prevalence was evaluated for some of these host species. 34.5% of the sampled birds were infected, all with low level parasitemias. *Haemoproteus* spp. was only present in migrant species and was not locally transmitted, while *Plasmodium* spp. infected more species and reached a higher overall prevalence. Prevalence differed among bird species and was affected by different variables for each species: it was associated with age in the Reed Warbler *Acrocephalus scirpaceus*, with season in the Cetti's Warbler *Cettia cetti* and with year in the House Sparrow *Passer domesticus*. Site did not influence prevalence for any species at this small geographical scale. Reed Warbler adults had already migrated to Africa and contacted with two different parasite faunas, whereas yearlings have not, thereby explaining the importance of age to explain parasitemia in this species. For the resident Cetti's Warbler, prevalence varied significantly with season, perhaps due to lower food availability in autumn and winter, making birds weaker and more prone to infection.

Keywords: Haemosporidiosis; avian malaria; *Plasmodium*; *Haemoproteus*; prevalence; reed bed passerines; South-Western Europe.

Introduction

Parasites can reduce their host's fitness by weakening the host, causing pathologies and even modifying the host's behaviour, including decreasing its physical and feeding activity (Valkiūnas 2005). They are a powerful selective force that influences and regulates hosts natural populations; as hosts fight back, both parasite infectivity and host resistance coevolve (Clayton and Moore 1997). Host-parasite interactions can be locally influenced by abiotic factors such as climate, season or habitat type, and by biotic effects such as host age or sex (Sol et al. 2003; Freeman-Gallant et al. 2001), causing each parasite's prevalence to vary throughout its distribution range. Local changes in the equilibrium of this system, such as climate changes or the introduction of new species, may originate the risk of disease outbreak (Atkinson and van Riper III 1991).

Birds are often infected by blood parasites of the genera *Plasmodium* and *Haemoproteus* (Apicomplexa: Haemosporida). Although

sometimes both genera are referred to as avian malaria parasites, strictly speaking, only *Plasmodium* causes avian malaria. Both infect birds through the bite of an infected dipteran vector, which can be a mosquito in the case of *Plasmodium* spp. or a *Culicoides* midge or a hippoboscid fly for *Haemoproteus* spp. (Valkiūnas 2005). After the transmission episode there is a pre-patent period during which the parasites reach the blood cells and then the infection's intensity in the blood stream (also called parasitemia) rises until it reaches a peak.

Among birds, the passerines can have relatively high prevalence of haemosporidians, although there are variations between host species and even between populations of different geographical areas (Valkiūnas 2005). For example, House Sparrows (*Passer domesticus*) showed a prevalence of 47% *Plasmodium* spp. in one French population and, in another population, of 75% *Plasmodium* spp. and 0.01% *Haemoproteus* spp. (Bonneaud et al. 2006). These geographical differences may arise from the sites' differences in habitat conditions, bird community (presence of alternative host species), vector abundance and vector activity, which influence parasite transmission (Pérez-Tris and Bensch 2005a). Also, each parasite species can have its own transmission season (winter, summer or year-round), causing variation in prevalence throughout the year (Pérez-Tris and Bensch 2005b); if the parasite community of all sites is not the same, this will add to the differences between sites. In migrant species, the observed prevalences in a particular site are also influenced by the conditions and parasite faunas encountered during the whole migration cycle. Such is the case of the Great Reed Warbler (*Acrocephalus arundinaceus*), that showed 10% *Haemoproteus* spp. and 10% *Plasmodium* spp. in Latvia, 21% *Haemoproteus* spp. in Germany (Bensch et al. 2000), 17% *Haemoproteus* spp. and 27% *Plasmodium* spp. in Sweden (Bensch et al. 2007), and 31% *Haemoproteus* spp. and 23% *Plasmodium* spp. in Bulgaria (Dimitrov et al. 2010).

Reed beds are relatively isolated patches of habitat, which can differ in their conditions and vector community, so they are expected to have a large geographic variation in their dynamics of parasite transmission. There is substantial information on haemosporidian infections in reed bed passerines, but this knowledge is not homogeneous across the breeding and the migration range. While a lot is known about the northern European populations (see all references in the paragraph above), there is little knowledge on their southern European breeding range (Fernandez et al. 2010; Merino et al. 2000b; Merino et al. 1997).

This study aimed to find which factors are associated with haemoparasite prevalence in several passerine species. Microscopy and molecular techniques were used to detect *Haemoproteus* and *Plasmodium* spp. infections in two species of sparrows (family Passeridae) and seven species of migrant and resident Old World warblers (four families of the superfamily Sylvioidea) at three different Portuguese reed beds along a small latitudinal gradient. The prevalence was analysed according to site, season and host's characteristics for four of these bird species: the Cetti's Warbler *Cettia cetti*, the Reed Warbler *Acrocephalus scirpaceus*, the Tree Sparrow *Passer*

montanus and the House Sparrow *Passer domesticus*.

Methods

Study area

Samples were collected in three Portuguese wetlands close to the coast: Taipal (N 40º11', W 8º41'), Santo André (N 38°4', W 8°48') and Vilamoura (N 37º04', W 8º07'), aligned along a latitude gradient of 370 Km. Santo André is a brackish water coastal lagoon, while the other two are freshwater marshes. These sites' vast reed beds (*Phragmites australis*) house a wide variety of ducks, waders and other water birds, both resident and migratory. They are important breeding sites for resident passerines like the Cetti's Warbler and also breeding and refuelling areas for migrants such as the Reed and the Great Reed Warblers. The still waters of the three study sites are good breeding areas for mosquitoes, which are abundant from May to September (pers. observation).

Field work

Passerines of nine species were sampled from March 2007 to November 2008 in all the sites. Three seasons were considered: breeding season (March to July), summer/autumn migration (August to September) and winter (October to January). The Eurasian Tree Sparrow, the House Sparrow and the Cetti's Warbler were residents, so could be sampled in all seasons; the Willow Warbler, a passing migrant, was mostly sampled in autumn; the Common Chiffchaff (*Phylloscopus collybita*) winters in the study area, so was only captured during autumn and winter. Four other species reproduce in these marshes and are absent during winter, so were only found during the breeding season and autumn: the Reed and the Great Reed Warblers, the Savi´s Warbler (*Locustella luscinioides*) and the Iberian Chiffchaff (*Phylloscopus ibericus*).

Individuals were captured with mist nets, ringed, weighted, measured and then aged according to Svensson (1992). A blood sample (around 40 µl) was collected from the jugular or brachial veins using a 25 G or 30 G needle, after which the birds were released. From that sample, a drop of blood was used to make a smear and the rest was stored in 96% ethanol for future DNA amplification. The smears were prepared according to Valkiunas (2005), air-dried and fixed as soon as they were dry in 96% ethanol for 3 minutes.

Laboratory work

Within two weeks from preparation, the smears were stained with a 10% solution of Giemsa's stain for 50 minutes (Valkiūnas 2005). Smears were screened for parasites with a light microscope. First the whole smear was examined under a 400x magnification, and then random fields were screened for parasites under a 1000x magnification until 20 fields (averaging 10.000 erythrocytes) were observed. Infection intensity was considered to be the number of detected parasites per 10.000 screened erythrocytes.

Total DNA was extracted from the blood samples using a standard ammonium acetate protocol. Birds were sexed by a polymerase chain reaction (PCR) amplifying a CDH gene's fragment, using the primers 0057F (3' CGTCAA TTTCCATTTCAGGTAAG 5') and 002R (3' TTATTGATCCATCAAGTCTC 5'). The reaction

products were run in 2% agarose gels for band visualization and sexing of each sample. Success in this reaction also confirmed that the extracted DNA was in good enough condition to be amplified by PCR.

Infections were diagnosed using a nested PCR protocol developed by Waldenström et al. (2004), targeted at a portion of the parasite's mitochondrial cytochrome *b* gene. The used primers were specific for *Haemoproteus* and *Plasmodium* spp.: HaemNF/ HaemNR2 (Waldenström et al. 2004) for the pre-amplification PCR, followed by HaemF/HaemR2 (Bensch et al. 2000) for the specific PCR. Each reaction had a total volume of 25 µl and included approximately 25 ng of genomic DNA, 1.5 mM $MgCl_2$, 2.5 µl 10x PCR buffer II, 400 mM of each deoxynucleoside triphosphates, 0.6 mM of each primer, and 0.625 U of AmpliTaq DNA polymerase (Applied Biosystems, Foster City, California). The thermal profile started with 3 min of denaturation at 94 ºC, followed by cycles of 94º C for 30 sec, 50º C for 30 sec, 72º C for 45 sec, and ended with an elongation step at 72º C for 10 min. The pre-amplification PCR ran for 20 cycles and the final PCR ran for 35 cycles (Waldenström et al. 2004). Final amplification products (479 bp) were run in a 2% agarose gel.

We controlled for contaminations by including a negative control per each 24 samples during extraction and a negative control (water) for each 8 samples during PCR. None of these controls ever showed amplification. 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 genus.

Statistical analysis

Birds were considered infected when they had a positive PCR or an infected smear (in the few cases (1.8%) in which PCR failed to detect infections that were confirmed in smears). All the infected birds were considered to be infected by one single parasite lineage, even in the five cases when co-infections of parasites of different genera were recorded by microscopy. The used PCR protocol frequently reads mixed infections of different haemosporidian species as if they were single infections, identifying only one of the present parasite lineages (Valkiūnas et al. 2006a). Therefore, in the cases of co-infections by *Haemoproteus* and *Plasmodium* spp., the prevalence of one of these genera was underestimated, but the fraction of infected individuals of each host species and the overall intensity of infection were not altered by this procedure.

Prevalence of Haemoproteus and Plasmodium spp.:

A log-linear analysis was performed to test the association of year, season and the sampling site with the presence or absence of infection (a variable that reflects prevalence, defined as infection status: 0 = bird not infected, H = bird infected with *Haemoproteus* spp. and P = bird infected with *Plasmodium* spp.). This analysis was carried out for the four most abundant bird species in the three study areas: Cetti´s Warbler, Reed Warbler, House Sparrow and Tree Sparrow.

The log-linear models were built using with STATISTICA 7.0 (Statsoft Inc. 2002) by a stepwise process. First, all k-factor interactions (k = 1, 2, etc.) between variables were tested simultaneously to find which order of interactions needed to be included to significantly explain the data. Then, the terms of that order or lower that significantly improved the model's fit were included one by one (Statsoft 2011). The fit of the models to the data was determined using maximum likelihood χ^2 tests (H_0: the model describes the variation in the data) and the accepted model for each host species was the least complex model that fitted the data.

Infection intensity:

For the four host species with a larger sample (Table 1), a general linear regression model was built to relate infection intensity with age, sex, site, season, year and body condition. These models were built including all effects in STATISTICA 7.0. The dependant variable was the $\log_{10}$ (intensity + 1). An intensity value of 0.5 was attributed to infections undetected by smear observation but with a positive PCR (considering that these samples have infections weaker than 1 parasite per 10.000 erythrocytes). Age, sex, site and year were seen as categorical predictors. The body condition, a continuous variable, measured the variability in weight that was not explained by the animal's body size. This parameter was the standard residual obtained from a linear regression between weight and wing length for each species.

Results

Prevalence of *Haemoproteus* and *Plasmodium* spp.:

1057 birds from nine species were sampled, out of which 365 (34.5%) showed infections: 5.8% by *Haemoproteus* spp., 26.9% by *Plasmodium* spp. and 1.8% were not identified (corresponding to 19 infections detected in the smears, but which could not be amplified by PCR). Prevalence varied considerably between bird species, from 58.8% for the Cetti's Warbler to zero for the Common Chiffchaff (Table 1).

There were significant differences between prevalence of infection in bird species of the same genus, except for *Passer* (for *Acrocephalus*, χ^2_1 = 8.02, p = 0.004; for *Phylloscopus*, χ^2_2 = 5.94, p = 0.05; but for *Passer*, χ^2_1 = 0.0004, p = 0. 985). Within the whole sample of infected birds, the number of infections by *Plasmodium* (82.3%) was significantly higher that the number of birds infected by *Haemoproteus* spp. (17.7%, χ^2_1 = 154.9, p < 0.001). Supplementary table S1 provides the complete list of all the lineages found in this study. The most prevalent lineage of parasites was SGS1, which caused 54.2% of all infections, including 91.7% of infections in the Cetti's Warbler, 6.3% in the Reed Warbler, 81.8% in the House Sparrow and 95.0% in the Tree Sparrow.

Haemoproteus spp. was only present in migrant species (χ^2_1 = 18.64, p < 0.001), with the single exception of one House Sparrow, a species known to spend limited time in the reed bed. Almost all the migrants infected with *Haemoproteus* spp. were adults; only two Willow Warblers sampled during migration in autumn

Table 1. Total sample size and infection prevalence for the nine host species.
Parasite genera are: P = *Plasmodium*, H = *Haemoproteus*, unid. = unidentified genus.

Bird species	**Sample size**	**Infected *(%)***	**Parasite genus**
Cetti's Warbler, *Cettia cetti*	245	144 *(58.8%)*	144 P
Great Reed Warbler, *Acrocephalus arundinaceus*	33	19 *(57.6%)*	12 H, 7 P
Reed Warbler, *Acrocephalus scirpaceus*	387	128 *(33.1%)*	46 H, 63 P, 19 unid
Savi's Warbler, *Locustella luscinioides*	45	7 *(15.6%)*	1 H, 6 P
Common Chiffchaff, *Phylloscopus collybita*	116	0	-
Iberian Chiffchaff, *Phylloscopus ibericus*	27	1 *(3.7%)*	1 P
Willow Warbler, *Phylloscopus trochilus*	36	2 (5.6%)	2 H
House Sparrow, *Passer domesticus*	114	44 (38.6%)	1 H, 43 P
Tree Sparrow, *Passer montanus*	54	20 (*37.0%*)	20 P
Total	1057	365 *(34.5%)*	62 H, 284 P, 19 unid.

Table 2. Best log-linear models reflecting the variables that influence infection status for each species. The tested variables were age, sex, season, site and year. Partial associations are computed by evaluating the gain of fit of the model that includes the corresponding interaction with the model that excludes it (Statsoft 2011).

Host species	**Influent variable**	**Variable's partial association**	**Final model's test of fit (Max. Lik. χ^2)**	**Effect of the selected variable in the infection status**
Cetti´s Warbler	Season	χ^2=11.63, p=0.003	χ^2=23.34, 23 d.f. p=0.441	Prevalence (of *Plasmodium* spp.) is higher in the reproductive season, lower in winter.
Reed Warbler	Age	χ^2=36.06, p<0.001	χ^2=102.85, 124 d.f. p=0.917	Prevalence increased with age, both for *Plasmodium* spp. and for *Haemoproteus* spp..
House Sparrow	Year	χ^2=7.497 p=0.006	χ^2=19.88, 22 d.f. p=0.590	Higher prevalence in 2007.
Tree Sparrow	None	- -	χ^2=5.18, 15 d.f. p=0,990	None of the tested variables affected infection status.

2007 were juveniles. Therefore, all three exceptions were birds that had certainly spent much time outside the study sites. This suggests that none of the *Haemoproteus* lineages was transmitted locally and that the infected birds probably acquired those parasites elsewhere. The *Plasmodium* genus was detected in resident species and/or in juveniles that were still attached to their birth reed bed, which proves local transmission of at least some lineages of this genus, such as SGS1.

The log-linear analyses suggested that different factors contribute to explain the infection status of each of the four tested bird species (Table 2). The prevalence in the Cetti's Warbler was affected by season, increasing from the reproductive season to autumn and maintaining the same higher levels of infection in winter (Fig. 1). This pattern was also present to some extent in the House Sparrow (Fig. 1). The prevalence in the Reed Warbler was affected by age (Fig. 2), increasing in adulthood both for *Plasmodium* spp. and for *Haemoproteus* spp.. For the House Sparrow, prevalence was associated with the year, showing differences between study years. Tree Sparrows did not show an effect of any of the tested variables. Site and sex were unimportant for all species. The same variables were elected when these models were redone excluding *Haemoproteus* infections, since their transmission was not affected by local conditions.

Intensity of infections

772 smears were examined, revealing 122 infections (Fig. 3). For the same birds, PCR results show 280 infections, suggesting that most of these parasitemias were lower than 1 parasite per 10.000 erythrocytes (i.e., below the threshold of detection by the used microscopy protocols). Infection intensity ranged from less than 1 to 291 parasites per 10.000 erythrocytes, which is considered a low level parasitemia (Zehtindjiev et al. 2008; Valkiūnas 2005).

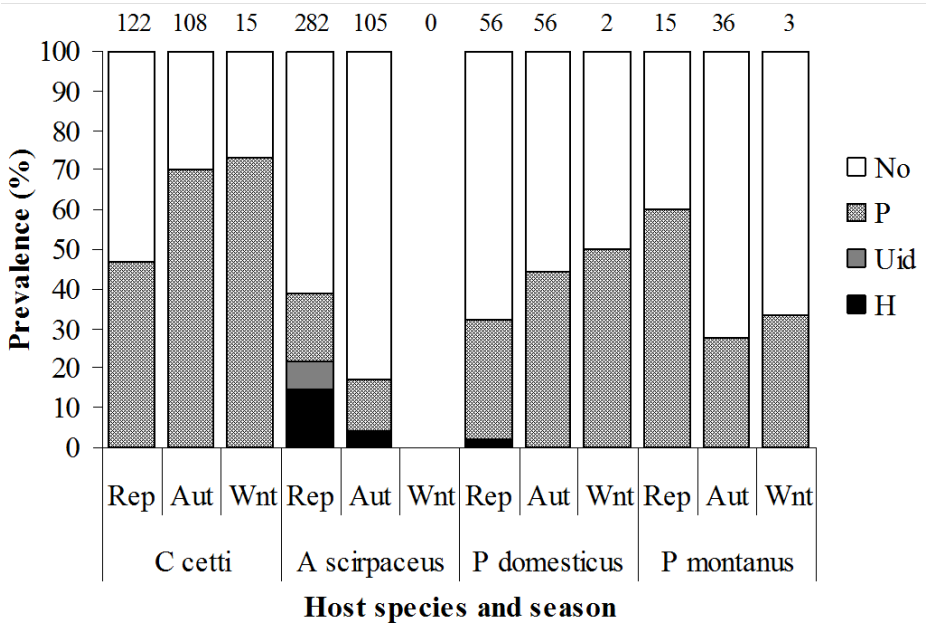

Fig. 1. Prevalence by season for the four species with a larger sample. Seasons are: Rep = reproductive season, Aut = autumn and Wnt = winter; infection states are: No = no infection, P = *Plasmodium*, Uid = unidentified genus, H = *Haemoproteus*. Sample size for each season and species is shown above each column. *A. scirpaceus* is absent from the study sites during winter.

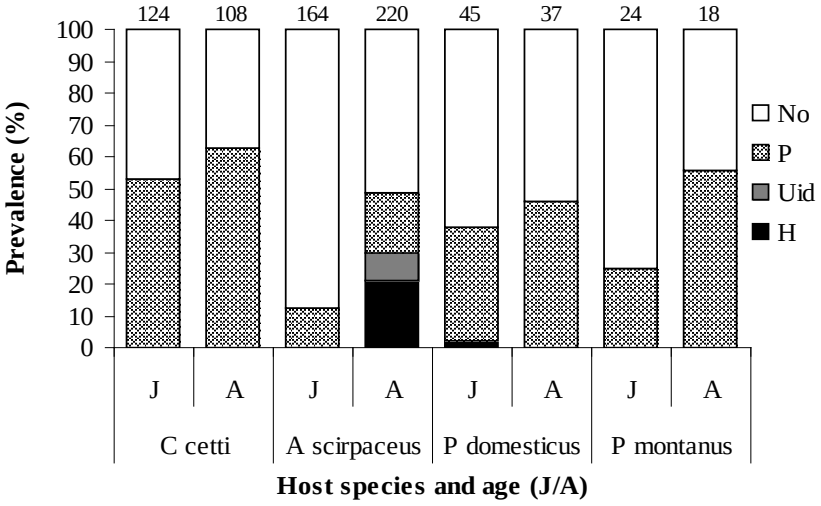

Fig. 2. Prevalence by age (J = juveniles, A = adults) for the four analysed species.

Infection states are: No = no infection, P = Plasmodium, Uid = unidentified genus, H = Haemoproteus. Sample size for each season and species is shown above each column.

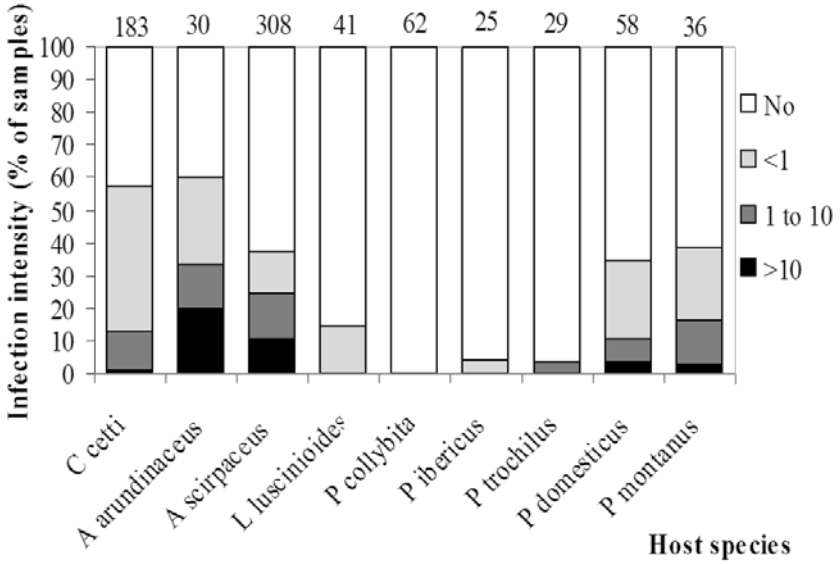

Fig. 3. Infection intensity in the nine bird species.

No = not infected, either by PCR or by smear observation. Within infections that were detected by PCR, there are three classes of intensity: less than 1 parasite/10.000 erythrocytes (infection detected by PCR, but not in smear); 1 to 10 parasites/10.000 erythrocytes; and above 10 parasites/10.000 erythrocytes. Sample size for each bird species is shown above its column (total sample size = 772).

For the 280 infected birds, infection intensity was related with the genus of the parasite: it was significantly higher for *Haemoproteus* than for *Plasmodium* spp., both for each species and for the overall set of samples ($\chi^2_2 = 111.17$, $p < 0.001$). For each of the four analyzed species, the general linear regression model built to explain infection intensity (not shown) included the same significant variable as the model that explained prevalence for that species. This was maintained when only *Plasmodium* spp. infections were considered.

Discussion

Prevalence:

The overall prevalence found in the studied bird species (34.5%) was within the range of previous observations in warblers and sparrows in

other areas, using similar detection techniques (Valkiunas et al. 2008 in Lithuania; Bonneaud et al. 2006 in France; Waldenstrom et al. 2002 in Nigeria). Prevalence in species of the same genus was significantly different for the warblers, but not for the sparrows. For birds of these genera, some studies have also found differences (Dimitrov et al. 2010; Pérez-Tris et al. 2007; Waldenström et al. 2002; Bensch et al. 2000), while others have not (Fernandez et al. 2010; Shurulinkov and Ilieva 2009). This indicates that prevalence is not phylogenetically determined and is probably more influenced by immune, physiological and ecological constraints.

There seems to be no transmission of *Haemoproteus* spp. in our study sites, although individuals infected elsewhere have active infections in their bloodstream. This agrees with the fact that *Haemoproteus* spp. main known vectors, the biting midges (genus *Culicoides*, Ceratopogonidae), prefer forested habitats and seem to be absent from the studied reed beds (R. Ventim, unpublished data). Transmission of some *Haemoproteus* lineages seems to occur exclusively in Africa (Hellgren et al. 2007a; Waldenström et al. 2002), while some *Plasmodium* parasites such as SGS1, a lineage of *Plasmodium relictum* (Palinauskas et al. 2007), seem to adapt better to several different transmission conditions (Hellgren et al. 2007b), so can be transmitted locally as well as in Africa.

The log-linear analysis showed that the overall prevalence depends on different variables for each of the studied bird species. For the Reed Warbler, the only analysed migrant, age was the most important factor. This is because the juveniles have only been exposed to the local parasites, whereas adult individuals have already migrated and been exposed to the parasite faunas of both their wintering and reproductive sites, which should explain their higher overall prevalence. This was obvious for *Haemoproteus* parasites, but may also happen with some African-transmitted *Plasmodium* lineages (Hellgren et al. 2007b). The remaining species, all residents, showed different patterns of effects. This means that every host species has a different susceptibility to infection, which can be mediated by differences in their vector attraction, exposure behaviours, immune system's reactions to environmental changes, etc. Also, different birds can be infected by distinct lineages, each with its own transmission rhythms and patterns that can be masked when the overall prevalence is analysed.

An increase in prevalence during the reproduction season could be expected due to several possible causes: 1) an increase of vector populations because of good temperature and humidity conditions; 2) a reduced host immunocompetence due to reproduction stress and energy investment (Schultz et al. 2010; Mendes et al. 2005), making the birds more susceptible to be infected; and 3) the spring relapse of latent infections, associated with the previous factor (Schultz et al. 2010; Valkiūnas 2005; Atkinson and van Riper III 1991). However, the season's effect was only significant for the Cetti's Warbler, in which the prevalence increased strongly from spring to autumn. The Cetti's Warbler body condition dropped at the end of summer, at the same time in which arthropod availability should have decreased in the reed beds. Therefore, the more stressful season of the year for resident birds might be autumn and winter, leaving them more

immuno-depressed and prone to infections than reproductive stress during the breeding season. Mosquitoes are abundant in these areas until late summer (September); the infections contracted at that time should manifest during autumn (after passing the pre-patent period; Palinauskas et al. 2008) and were probably maintained during winter, while the birds' physical condition continued to be poor. This can explain why prevalence was higher during autumn and winter for resident bird species, even though there were much less mosquitoes in those seasons (R. Ventim, unpublished data). This tendency should be more obvious in more specialized species, like the Cetti's Warbler, and more subtle in resident generalists such as the House Sparrow, since generalists can also rely on other food sources.

The location did not influence prevalence patterns in this small geographic scale. The reed beds of our study are geographically isolated patches and therefore they were expected to have somewhat different communities of hosts and vectors, generating differences in the transmitted parasites (Pérez-Tris and Bensch 2005b). However, the bird communities in these patches are probably connected through the juvenile dispersal movements of their residents, stopping-over of migrants that have nested in different locations and the contact between migrant populations of different origins in their wintering grounds. These bird movements, together with similar vector communities, apparently are enough to make the parasite communities similar in all the studied sites. Other studies have found significant spatial differences in prevalence for breeding populations of many bird species; however, those studies were conducted at a wider geographical scale (Bensch and Akesson 2003 in Sweden; Bennett et al. 1995 in Scandinavia; Merilä et al. 1995 throughout Europe), compared different habitats (Loiseau et al. 2010 in Ghana) or compared sites at different altitudes (Shurulinkov and Ilieva 2009 in Bulgaria).

Intensity of infections:

All the infected birds that were sampled had low level parasitemias, consistent with the chronic phase of infection (Zehtindjiev et al. 2008; Valkiūnas 2005). Heavily infected birds, at the peak of their infection, are seldom sampled using mist nets because they should be weakly mobile or less active than healthy individuals (Valkiūnas 2005). Because in practice this group of individuals is not covered by our sample, the actual prevalence of haemoparasites in the wild should be somewhat higher than what can be found using this sampling method. The parasitemia of *Haemoproteus* spp. infections was significantly higher than that of *Plasmodium* spp.. This confirms the findings of Atkinson and van Riper III (1991), even at the chronic stage of infection.

In the four analysed host species, the body condition was not related to infection intensity or to the presence/absence of infection. This agrees with other studies of experimental infections (Valkiūnas et al. 2006b; Garvin et al. 2003) and natural infections in several species (Schultz et al. 2010; Edler et al. 2004; Bennett et al. 1988). However, this kind of relationship is hard to detect in wild animals because of the afore-mentioned under sampling of acutely infected birds, which are those that might show a depressed body condition (Valkiūnas 2005).

This was the first study of haemosporidian transmission in SW Europe including a community of nine passerine species. Extensive sampling of this kind adds to the accumulated knowledge of avian malaria infections, helping to appreciate presence and prevalence differences between distinct geographic regions or environmental conditions.

Acknowledgments

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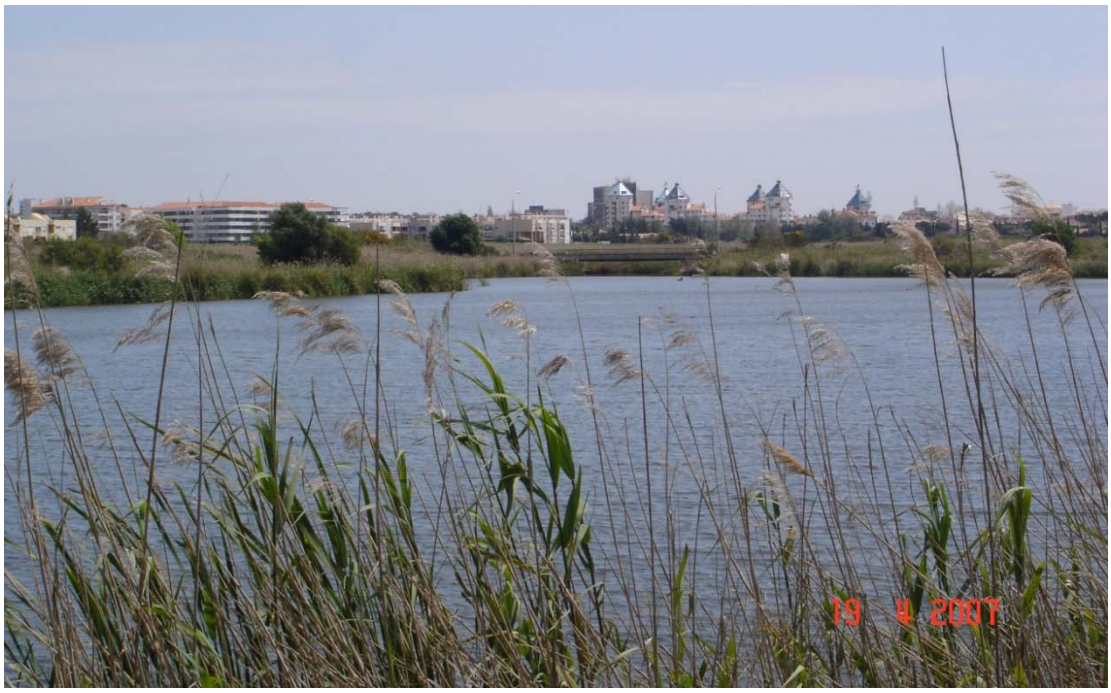
Supplementary Table S1. Parasite lineages infecting each bird species in this study. Lineages were identified by comparing the cyt-b sequences with the ones in GeneBank and MalAvi (Bensch et al. 2009).

Bird species	*Haemoproteus* lineages	*Plasmodium* lineages
Cetti's Warbler, *Cettia cetti*	-	PADOM12, SGS1, SYAT05.
Great Reed Warbler, *Acrocephalus arundinaceus*	GRW01, GRW16.	GRW02, GRW04, GRW17, SGS1.
Reed Warbler, *Acrocephalus scirpaceus*	HIPOL1, MW1, RW1, SW1.	GRW04, GRW06, PADOM12, RTSR1, SGS1, SW2, SW5.
Savi's Warbler, *Locustella luscinioides*	MW1.	COLL1, GRW04, GRW06, WW4.
Common Chiffchaff, *Phylloscopus collybita*	-	-
Iberian Chiffchaff, *Phylloscopus ibericus*	-	BT6.
Willow Warbler, *Phylloscopus trochilus*	WW1, WW2.	-
House Sparrow, *Passer domesticus*	PADOM23.	PADOM01, PADOM12, SGS1.
Tree Sparrow, *Passer montanus*	-	SGS1.

CHAPTER 2

Host-parasite associations and host-specificity in haemoparasites of reed bed passerines

Rita Ventim, Joana Morais, Sara Pardal, Luísa Mendes, Jaime A. Ramos and Javier Pérez-Tris



Ventim R, Morais J, Pardal S, Mendes L, Ramos JA, Pérez-Tris J (in press) Host-parasite associations and host-specificity in haemoparasites of reed bed passerines. Parasitology.

Vilamoura’s reed bed.
Photo by: Rita Ventim (2007).

Host-parasite associations and host-specificity in haemoparasites of reed bed passerines

Abstract

The host specificity and host sharing of avian haemoparasites (genera *Haemoproteus* and *Plasmodium*) is still poorly known, although they infect a large proportion of several studied bird populations. This study used molecular techniques to detect haemoparasites in marsh warblers and in other passerines that feed in reed beds, at four sites in Portugal. The host-specificity of the parasite lineages was analyzed and compared with other cases described in the literature to assess whether apparent host specificity changes according to the studied system. Nine lineages of *Haemoproteus* and 15 of *Plasmodium* were found, of which only ten *Plasmodium* were proven to have local transmission. Each lineage was confined to a distinct set of host species. The distribution of parasites in the host species was non-nested, meaning that specialist lineages did not always share hosts with generalists. The most prevalent lineages were those with a wider host range, indicating that the ability to infect more hosts will enhance a parasite's prevalence in its entire host range. We also found that in our areas, a specialist parasite (*H.* MW1) appears to have a more generalist character than described in the literature, suggesting that a parasite's apparent specialization can depend on the type of host species that are sampled.

Keywords: Avian malaria; Haemoproteus; haemosporidian; host–parasite association; host range; local transmission; nestedness; Plasmodium; specialists versus generalists.

Introduction

Parasites obtain food, habitat and dispersal from their hosts (Valkiūnas 2005). A generalist parasite is one that is capable of infecting and completing its life cycle in many host species, while a specialist will be found in only a few host species. Specialist parasites may be very well adapted to particular hosts, but will be unable to infect other closely related species if they become in contact with them.

A parasite's probability of infecting a suitable host depends on many factors, including the host-parasite compatibility and rate of encounter (Combes 1997). The probability of physical contact with a susceptible host is influenced by host and parasite's behaviour, life-cycle, population density, etc. Vector-transmitted parasites have complex systems of interactions, which also include the vector's behaviour and population dynamics. If the vectors contact with many possible host species, then the parasites present in the vector might end up in incompatible

or sub-optimal hosts, which reduces the probability of successful infections (Dobson 2004). Therefore, a vector-transmitted parasite in a host-rich community has advantages in being host-generalist, that is, maintaining compatibility with a wide set of hosts, even if some of them are not optimal. This should increase its encounter rate with suitable hosts and, therefore, its overall prevalence in the community (Hellgren et al. 2009; Dobson 2004).

But does a parasite always appear as a specialist or as a generalist, or will parasites be considered more or less generalist according to the conditions that they face? In different parts of their distribution range, parasites will find different assemblages of possible hosts, vectors and even other competitor parasites. According to the different communities that they find, they might appear to be more or less host-specialist. A parasite that is a generalist in one community may be unable to infect most of the hosts present in a different place, thus appearing to be more specialist; and a parasite that is fully adapted to few hosts may encounter a new community of naive hosts and be able to infect many of them, therefore becoming a generalist in that community.

At a community level, the interactions between parasites and their hosts define an antagonistic network. Determining the general ecological pattern of these interactions may help to understand and predict the spread of parasites and diseases in general (Graham et al. 2009). Nestedness is a particular structure reported for many networks, in which specialists only interact with subsets of the species that interact with more generalist organisms. The nested pattern has been described for many mutualistic webs (Bascompte and Jordano 2007; Bascompte et al. 2003) and, although it has been suggested that it should not apply to most antagonistic networks (Thompson 2006), nestedness was also found in host-ectoparasite networks (Graham et al. 2009). Applied to host-parasite networks, a nested pattern would mean that specialist parasites should only be able to infect a few of the host species that generalist parasites can infect – or, in other words, that more resistant hosts would only be infected by a few generalist parasites, while the less resistant hosts would be infected both by generalist and specialist parasites (Graham et al. 2009).

Protists of the genera *Plasmodium* (also referred to as avian malaria parasites) and *Haemoproteus* (Apicomplexa: Haemosporida) infect the blood cells of birds through the bite of an infected dipteran vector – a mosquito in the case of *Plasmodium* spp., a biting midge or an hippoboscid fly for *Haemoproteus* spp. (Valkiūnas 2005). The use of molecular techniques (Bensch et al. 2004; Waldenström et al. 2004; Ricklefs et al. 2004) has unveiled that this is a very diverse group and has defined mitochondrial lineages, which greatly outnumber the traditional morpho-species and may be considered as separate species (Pérez-Tris et al. 2007; Bensch et al. 2004). At the lineage level, the host specificity of haemosporidians is still poorly understood, although at the genus level, the *Plasmodium* genus seems to contain more generalist parasites than *Haemoproteus* (Fallon et al. 2005). In both genera, while some lineages infect hosts from a wide range of families, others are very host-specific (Waldenström et al. 2002). The structure of these host-endoparasite interaction networks is also unknown. Passerine species are known to suffer from relatively high haemoparasite

infection rates, but these vary greatly between host species and between geographical areas (Valkiūnas 2005).

This study focused on the presence of *Haemoproteus* and *Plasmodium* lineages in bird assemblages [two species of sparrows (family Passeridae) and seven species of Old World warblers (four families of the superfamily Sylvioidea)] at four reed beds in Portugal. The bird's parasite fauna was analyzed using molecular techniques. The structure of the host-parasite interaction network was assessed and the host specificity of each parasite lineage was compared with other cases reported in the literature. Overall, this study evaluated the degree of specialization of haemoparasite lineages in a rich community of bird hosts.

Materials and methods

Study area

This study took place in four Portuguese wetlands: Taipal (N 40º11', W 8º41'), Tornada (N 39º27`, W 09º3`) Santo André (N 38°4', W 8°48') and Vilamoura (N 37º04', W 8º07'). Populations of several species of mosquitoes, possible vectors of avian haemosporidians, reproduce here. All four wetlands have vast extensions of common reed bed (*Phragmites australis*), which attracts a wide variety of ducks, waders and other waterbirds, both resident and migratory. They are important breeding and refuelling areas for migrating passerines such as the Reed Warbler (*Acrocephalus scirpaceus*), the Great Reed Warbler (*Acrocephalus arundinaceus*) and the Savi's Warbler (*Locustella luscinioides*) and also harbour important populations of resident passerines, such as the Cetti's Warbler (*Cettia cetti*).

Field work

Passerines were captured with mist nets from March 2007 to November 2008 in all areas and from July to September 2009 in the Tornada site only. The most abundant species in these sites were sampled: the Reed and the Great Reed Warblers, the Savi's Warbler, the Willow Warbler (*Phylloscopus trochilus*), the Common and the Iberian Chiffchaffs (*Phylloscopus collybita* and *P. ibericus*), the Cetti's Warbler, the Eurasian Tree Sparrow (*Passer montanus*) and the House Sparrow (*Passer domesticus*, although this sparrow spends a great part of the day outside the reed bed). The two sparrows and the Cetti's Warbler are residents, the Willow Warbler is a passage migrant, the Common Chiffchaff winters in the study area and all the other species reproduce in these Portuguese marshes. Less abundant species that were present (of finches, thrushes, tits, warblers, etc.) were not sampled.

Individuals were ringed, weighted, measured and then sexed and aged according to Svensson (1992). A blood sample (around 40 µl) was collected from the jugular or brachial veins using a 25 G or 30 G needle and stored in 96% ethanol, after which the birds were released.

Laboratory work

Total DNA was extracted using a standard ammonium acetate protocol. Birds were sexed by a polymerase chain reaction (PCR) amplifying a CDH gene's fragment, using the primers 0057F

(CGTCAATTTCCATTTCAGGTAAG) and 002R (TTATTGATCCATCAAGTCTC). Resulting products were run in 2% agarose gels for band visualization. The successful sexing of a sample confirmed that the extracted DNA was in good enough condition to be amplified by PCR.

Samples were diagnosed for haemoparasite infections using a nested PCR developed by Waldenström et al. (2004). A portion of the parasite's mitochondrial cytochrome *b* gene was amplified using the primers HaemNF/HaemNR2 (for pre-amplification) followed by HaemF/HaemR2 (Bensch et al. 2000), which are specific to *Haemoproteus* and *Plasmodium* spp. Each PCR included approximately 25 ng of genomic DNA, 1.5 mM $MgCl_2$, 2.5 µl of 10x PCR buffer II, 400 mM of each deoxynucleoside triphosphates, 0.6 mM of each primer, and 0.625 U of AmpliTaq DNA polymerase (Applied Biosystems, Foster City, California), in a total volume of 25 µl. The thermal profile started with 3 min of denaturation at 94 ºC, followed by cycles of 94º C for 30 sec, 50º C for 30 sec, 72º C for 45 sec, and ended with an elongation step at 72º C for 10 min. 1 µl of the products of the pre-amplification PCR was used as template for the second PCR. This final reaction used the same reagents in the same concentrations and the same thermal profile, the only difference being that the pre-amplification PCR ran for 20 cycles while the final PCR ran for 35 cycles (Waldenström et al. 2004). Final amplification products (479 bp) were run in a 2% agarose gel.

We controlled for contaminations by including a negative control per each 24 samples during extraction and a negative control (water) for each 8 samples during PCR. None of these controls ever showed amplification. Samples that were negative for infection were confirmed by a second nested PCR, while all 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 genus and lineage. New parasite lineages and new host-parasite associations were confirmed by repeating the whole process.

Data analysis

In order to test for a nested distribution of parasite lineages in the host species, a matrix of presences/absences of all lineages in all host species was built and then ordered from the more parasitized hosts (lines filled with more presences) to the less parasitized hosts (less filled lines). The matrix's nestedness metric NODF (Nestedness based on Overlap and Decreasing Fill; Almeida-Neto et al. 2008) was calculated using ANINHADO 3 (Guimarães and Guimarães 2006). The same software performed a nestedness analysis, which compares the filling structure of our matrix with the structure of a random matrix. Nestedness was tested for using χ^2 tests against the null hypothesis that the parasites present in less parasitized hosts are a sub-set of the parasites in more infected hosts. The program's CE null model builds random matrixes by filling cells in proportion to the row and column totals of each lineage and species, against which our matrix was compared (Graham et al. 2009). This analysis was

made first for each site, then for all birds from the four locations pooled together.

Each parasite's host-specificity was calculated considering the number of host species it could infect, the prevalence in all the infected species and also the taxonomic distance between such hosts. Two different specificity indexes were calculated: the host breadth index, HB (Fallon et al. 2005) and the standardized host range index, S_{TD*} (Poulin and Mouillot 2005). With both indexes, low values indicate parasite lineages that primarily infected closely related hosts, while high values reflect parasite lineages that were found across divergent host species. The higher the indexes are, the more generalist is the parasite. However, the two indexes have slightly different behaviours.

The HB is based on the phylogenetic distance between a parasite's hosts, weighted by the parasite's prevalence in the different hosts (Fallon et al. 2005):

$$HB = \sum_{i=1}^{n} \sum_{j=1}^{n} \omega_{ij} (p_i p_j)$$

p_i and p_j being the prevalence of the parasite in host species i and j. Because not all the phylogenetic distances between hosts were available, in this study they were replaced by the taxonomic distinctness (Clarke and Warwick 1998): ω_{ij} is the number of taxonomical steps (from the species to the genera, family, infra-order or order level) needed to get to the common ancestor of any pair of hosts. This modification also allows a more direct comparison with the S_{TD*} index, which originally uses taxonomic distinctness (Poulin and Mouillot 2005; Clarke and Warwick 1998). For parasites with only one host (i), the HB was calculated as p_i^2. This index increases whenever the number of host species increases, but can be greatly influenced by the prevalences in each host, giving out very different results for parasites with similar number of hosts (for example, for parasites with only one host species).

The S_{TD*} (Poulin and Mouillot 2005) shows the mean taxonomic distinctness among the host species used by a parasite, weighted for the parasite's prevalence in the different hosts:

$$S_{TD*} = \frac{\sum_{i=1}^{n} \sum_{j=1}^{i<j} \omega_{ij} (p_i p_j)}{\sum_{i=1}^{n} \sum_{j=1}^{i<j} p_i p_j}$$

p_i and p_j being the prevalence of the parasite in host species i and j and ω_{ij} the taxonomic distinctness between two host species. Whenever there was only one host, the S_{TD*} was considered to equal one. This index has a narrower variation range, allowing easier interpretation of values, and is more stable when hosts with similar number of hosts are compared; but it does not necessarily grow as the number of hosts increases (the addiction of closely related hosts will actually lower the index, by reducing the average distinctness between hosts).

To assess whether parasites always show the same degree of apparent host specificity in all the studied systems, we compared our own data with previously reported cases. For each parasite lineage found in this study, a list of prevalences in all the reported passerine hosts was compiled from the MalAvi database (Bensch et al. 2009). We assume that the sampling effort was the same for all parasite lineages in the total tested individuals, as all lineages can potentially be detected every time a blood sample is analyzed by this method. However, the sampling effort for all host species is unavoidably not constant across all the consulted

studies, which is a frequent problem in comparative studies.

Assemblages of nine bird species were simulated: a subset of nine birds was randomly selected from the compiled host list and each parasite's host range index was calculated for that subset of hosts. In this way, all the selected hosts had at least one parasite, but some parasites could be absent from all nine hosts (in this case, their indexes were considered to be zero). This simulation was repeated 1000 times. The probability of a lineage appearing to be more specialist in this particular system than is generally described is the probability of finding lower indexes in these simulations than in the real case under study.

Results

Prevalence of *Haemoproteus* and *Plasmodium*

1166 birds from nine species were sampled (Table 1), out of which 367 (31.5%) revealed infections (5.6% by *Haemoproteus* spp. and 25.9% by *Plasmodium* spp.). However, infection rates varied considerably between species, from 55.7% for the Cetti's Warbler to zero infections for the Common Chiffchaff. These nine bird species hosted 24 parasite lineages, 9 of *Haemoproteus* and 15 of *Plasmodium* (Table 2). Two lineages of *Haemoproteus* and two of *Plasmodium* were identified for the first time: *H.* GRW16 and *P.* GRW17 in the Great Reed Warbler, *H.* PADOM23 in a House Sparrow and *P.* CET01 in a Cetti´s warbler (GenBank accession numbers HQ262948 to HQ262951); these were named following the guidelines proposed by Bensch et al. (2009). Only three mixed infections were detected: one of *P.* PADOM01 and an unidentified *Plasmodium* lineage (in a House Sparrow) and two of a pair of unidentified *Plasmodium* lineages (one in a Cetti's Warbler, the other in a Reed Warbler). This is surely an underestimation of the real number of mixed infections, which is a known limitation of the used technique (Valkiūnas et al. 2006a).

Table 1. Sample size of all bird species and number of detected infections in each species.

Bird species	**Sample size**	**No. infections**	**% infections**
Cetti's Warbler, *Cettia cetti*	309	172	55.66
Great Reed Warbler, *Acrocephalus arundinaceus*	37	20	54.05
Reed Warbler, *Acrocephalus scirpaceus*	421	104	24.70
Savi's Warbler, *Locustella luscinioides*	46	7	15.22
Common Chiffchaff, *Phylloscopus collybita*	116	0	0.00
Iberian Chiffchaff, *Phylloscopus ibericus*	27	1	3.70
Willow Warbler, *Phylloscopus trochilus*	36	2	5.56
House Sparrow, *Passer domesticus*	121	45	37.19
Tree Sparrow, *Passer montanus*	53	16	30.19
Total	**1166**	**367**	**31.48**

Table 2. Number of infections of each parasite lineage found in each host species, followed by the host range indexes HB and S_{TD*} for each lineage.

H. GRW16, *H.* PADOM23, *P.* GRW17 and *P.* CET01 were found for the first time.

Host species are: *A aru = A. arundinaceus*, *A sci = A. scirpaceus*, *C cet = C. cetti*, *L lus = L. luscinioides*, *P ibe = Phylloscopus ibericus*, *P troc = P. trochilus*, *Pa do = Passer domesticus*, *Pa mo = P. montanus.*

	Lineage	*C cet*	*A aru*	*A sci*	*L lus*	*P ibe*	*P tro*	*Pa do*	*Pa mo*	HB	S_{TD*}
Haemoproteus	**GRW01**		12							$1.1x10^{-1}$	1
	GRW16		1							$7.3x10^{-4}$	1
	HIPOL1			1						$5.6x10^{-6}$	1
	MW1			40	1					$2.6x10^{-2}$	3.00
	PADOM23							1		$6.8x10^{-5}$	1
	RW1			5						$1.4x10^{-4}$	1
	SW1			2						$2.3x10^{-5}$	1
	WW1						1			$7.7x10^{-4}$	1
	WW2						1			$7.7x10^{-4}$	1
Plasmodium	**BT6**					1				$1.4x10^{-3}$	1
	CET01	1								$1.0x10^{-5}$	1
	COLL1				1					$4.7x10^{-4}$	1
	GRW02		1							$7.3x10^{-4}$	1
	GRW04		3	25	2					$8.0x10^{-2}$	2.12
	GRW06			12	1					$6.2x10^{-3}$	3.00
	GRW11	8		2				5		$1.6x10^{-2}$	3.91
	GRW17		1							$7.3x10^{-4}$	1
	PADOM01							2		$2.7x10^{-4}$	1
	RTSR1			3						$5.1x10^{-5}$	1
	SGS1	159	2	9				37	16	4.7	3.35
	SW2			1						$5.6x10^{-6}$	1
	SW5			3						$5.1x10^{-5}$	1
	SYAT05	3								$9.4x10^{-5}$	1
	WW4				2					$1.9x10^{-3}$	1

Most of the host-parasite associations found in this report had already been described in previous studies (Bensch et al. 2009 and references therein), except for six parasites in the Reed Warbler and for the newly identified lineages. One lineage of *Haemoproteus* (MW1) and four of *Plasmodium* (GRW04, GRW06, GRW11 and SGS1) infected more than one host.

Haemoproteus lineages were only present in migrant species, with one exception: one adult House Sparrow, a species known to spend limited time in the reed bed. Almost all the migrants infected with *Haemoproteus* spp. were adults; only two Willow Warblers sampled during migration, in autumn 2007, were juveniles. This suggests that *Haemoproteus* lineages are not transmitted locally and that the infected birds probably acquired the parasite elsewhere. In the *Plasmodium* genus, there were also some lineages that were only present in adult individuals of migratory species, hinting for non-local transmission: GRW4, GRW6, RTSR1 and WW4. On the other hand, lineages COLL1, GRW11, GRW17, SGS1, SW2, SW5 and SYAT05 occurred in resident species and/or were detected in juveniles that were still attached to their birth reed bed, which shows local transmission.

Lineage host specificity

The nestedness analysis revealed a non-random and non-nested pattern of parasites in each host, for each site as well as for the four areas pooled together (Coimbra: NODF = 6.74, null model's NODF = 10.05, p = 0.80. Santo André: NODF = 11.62, null model's NODF = 14.09, p = 0.68. Vilamoura: NODF = 6.13, null model's NODF = 9.15, p = 0.73. Tornada: NODF = 2.56, null model's NODF = 5.78, p = 0.81; all areas: NODF = 20.61, null model's NODF = 19.70, p = 0.39).

The parasites in the studied community had HB indexes between 5.6×10^{-6} and 4.7, and S_{TD*} indexes between 1 (when only one host species was found) and 3.91 (Table 2). When compared with the host range indexes obtained from MalAvi with Monte Carlo simulations, the lineage *H.* MW1 appeared as significantly more generalist in our study than in studies reported in MalAvi: the probability of finding a smaller index in the random simulations than in the studied system was 0.028 using HB and 0.049 using S_{TD*}. Two other lineages, BT6 and WW4, also showed as significantly more generalist in the studied system with the HB index (p = 0.005 and 0.002, respectively). Because these lineages only had one host species in this study, they did not give significant results with STD* (the significant result with HB being due only to higher prevalences in our study than in the simulations).

Discussion

Haemoproteus lineages were mostly found in migrants, and almost always in adults, with three exceptions: one resident House Sparrow and two juvenile Willow Warblers during their post-breeding migration. All these individuals had certainly spent plenty of time outside the studied reed beds and could have been infected elsewhere. This suggests that there is no transmission of *Haemoproteus* lineages in our study areas. However, this is not the case for other European areas; for example, transmission of *H.* WW2 to the Willow Warbler has been proved to happen in Swedish woodlands (Bensch and Akesson 2003). This agrees with the fact that *Haemoproteus* main known vectors, the biting midges (genus *Culicoides*, Ceratopogonidae), prefer forested habitats and seem to be absent from the studied reed beds (R. Ventim, unpublished data). Also, *Haemoproteus* spp. appears to have high affiliation to a single transmission area and a single bird

fauna, despite the vast numbers of infected birds that perform annual migrations between Africa and Europe (Hellgren et al. 2007b). Therefore, it is not expected that African transmitted *Haemoproteus* lineages would be able to adapt to the European conditions and vectors and be able to be transmitted to new hosts in their breeding quarters. *Plasmodium* parasites do this more often, as is the case for SGS1 (Hellgren et al. 2007b); so some of the *Plasmodium* lineages that were present in this community are expected to be transmitted locally as well as in Africa. Local transmission of *Plasmodium* lineages COLL1, GRW17, GRW11, SGS1, SW2, SW5 and SYAT05 was proven in our studied reed beds, because these parasites were found in birds that should have spent most of their lives in those areas (resident species or juveniles from migrant warbler species, all still attached to their birth reed bed).

Parasite distribution in the different hosts was not random, indicating that there are specific host preferences for each lineage. This agrees with the fact that most lineages in this study were only detected in one of the analyzed bird species, supporting our assumption that these are relatively specialized parasites. Nestedness was not detected in this interaction network. In a nested matrix, specialist parasites would concentrate in the most parasitized species of birds, sharing their hosts with generalist parasites (Bascompte and Jordano 2007; Bascompte et al. 2003). Since our matrix is not nested, in our case specialists do not always share hosts with generalist parasites, so they are free from the competitive pressure of generalists. This happened with BT6, WW1 and WW2, lineages that appeared as specialists in the matrix and were present in bird species that were not infected by generalist haemoparasites (the Iberian Chiffchaff and the Willow Warbler). These findings are the opposite from what was found by Graham et al. (2009) for ectoparasite-vertebrate host networks in general; this large-scale study analyzed networks of mosquitoes, lice, mites, ticks and fleas and mammals, birds, reptiles, amphibians and fish. It found nested structures, meaning that specialized ectoparasites interact with hosts that attract many parasites, while generalist parasites interact with these hosts as well as those that attract fewer parasites. This structure does not seem to apply to all host-parasite interaction networks.

Parasite lineages with higher overall prevalence in the bird community were those infecting a greater number of host species. Moreover, parasites with a broad host range reached high prevalence over a greater number of species, as was also found by Ricklefs et al. (2005) and Hellgren et al. (2009). Such lineages will be transmitted to vectors more often and, if they are host generalist, a higher proportion of the vectors' blood meals will end up in successful transmission. The encounter rate between these parasites and all species in the bird community increases, leading to higher prevalence in all of the hosts. Hence, the prevalence in each host species is amplified due to a wide host range (Hellgren et al. 2009).

The most prevalent of all lineages was *P.* SGS1, a lineage of the morpho-species *Plasmodium relictum* (Palinauskas et al. 2007). This parasite is known to be very host generalist, infecting hosts from over a dozen different families in distinct continents (Bensch et al. 2009 and references therein, such as: Hellgren et al. 2007; Beadell et al. 2006). In our study, it had a high

prevalence in the domestic sparrow and Cetti's Warbler, but was not as successful infecting Reed and Great Reed Warblers, two hosts that had high infection rates by other parasite lineages. These host species had already been described to be infected with SGS1 at similarly low prevalences (Dimitrov et al. 2010; Zehtindjiev et al. 2008; Waldenström et al. 2002). This suggests that these are not optimal hosts and that even a generalist parasite can have limited success infecting some hosts.

H. MW1 appeared to be significantly more generalist in our study areas than in most studies reported in MalAvi. Until now, MW1 had only been found in three *Acrocephalus* species (Krizanauskiene et al. 2006; Waldenström et al. 2002), even when many other host families were sampled concurrently. The present study found that it is also capable of infecting the Savi's Warbler, a host from a different genus and family that had not been sampled in the previous studies. This lineage seems to have narrow habitat preferences, concentrating in marsh warblers; therefore, it appeared to be more generalist in the studied reed bed communities than in studies (or simulations) involving hosts from other habitats. This exemplifies how the apparent specialization can sometimes depend on the type of host species that are sampled. More research is needed in order to discover more host-parasite associations and thus unveil more details of these complex interaction systems.

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CHAPTER 3

Do migratory routes of birds homogenize endoparasite assemblages?

Rita Ventim, Javier Pérez-Tris, Luísa Mendes, and Jaime A. Ramos

Reed Warbler (left) and Great Reed Warbler (wright).

Photos by: Rita Ventim (2007) and Simon Valle (Antikythira Bird Observatory, 2011)

Do migratory routes of birds homogenize endoparasite assemblages?

Abstract

In migratory birds, the local adaptation to parasite communities has been suggested to present a selective pressure strong enough to maintain the host's migratory connectivity. This can only happen if there are geographical differences in parasite communities. This study compared the haemosporidians (genera *Haemoproteus* and *Plasmodium*) infecting Reed Warblers and Great Reed Warblers (*Acrocephalus scirpaceus* and *A. arundinaceus*) found in different European sites, to see if haemoparasite assemblages are geographically structured along the two main European migration routes, the eastern and western flyways. Blood samples from 458 birds were collected in four sites in Portugal to represent the Western flyway, while the eastern flyway was represented by four sites taken from the literature (in Sweden, Bulgaria, Romania and Russia). 32 lineages were reported for the whole of Europe. Of these, 14 were exclusively found in the eastern flyway, 3 were exclusive of the western sites and the remaining 15 occurred in both flyways. When sites were compared two by two, the similarity of the site's parasite assemblages was not higher than random and could not be attributed solely to the flyway the sites belonged to. Therefore, in these host species, haemoparasite assemblages show little geographical structure throughout Europe. Because parasites are not geographically structured in a predictable way, host migratory connectivity will hardly evolve as the outcome of selection pressures related to haemoparasite avoidance.

Keywords: *Acrocephalus scirpaceus*; *Acrocephalus arundinaceus*; *Haemoproteus*; migratory connectivity; parasite assemblages; *Plasmodium*.

Introduction

Many migratory birds are faithful to both their breeding and their wintering sites (Newton 2008). Migratory connectivity is the extent to which individuals from the same breeding area migrate to the same non-breeding area, and vice versa. When connectivity is strong, most individuals from one breeding population will migrate to the same winter location, possibly leading to population structuring (Webster et al. 2002). It has been suggested that migratory connectivity is advantageous to birds because it contributes to lower their parasite loads (Møller and Szép 2011). In the coevolutionary race, adaptation of hosts to local parasites and of parasites to local hosts occurs. This local adaptation stabilizes host-parasite interactions and may lead to geographically localized host populations carrying distinct parasite assemblages (Kaltz and Shykoff

1998). Migratory birds should always be exposed to different parasite faunas in their breeding and wintering grounds, thus having to adapt to both parasite sets; even so, the more they disperse, the more they risk encountering different parasites. In this way, individuals that disperse and hence contribute to disrupt connectivity should encounter novel parasites to which they are not adapted, which will contribute to reduce their fitness (Møller and Szép 2011). In this scenario, the parasite communities of hosts with strong migratory connectivity can be expected to present geographical structuring in both the wintering and the breeding quarters of those hosts.

Haemoparasites of the genera *Haemoproteus* and *Plasmodium* (protists transmitted by the byte of a dipteran vector) are distributed all over the world (except on Antarctica) and in birds of almost all families (Valkiūnas 2005); however, some genetically distinguishable lineages tend to be geographically localized (Beadell et al. 2006; Kimura et al. 2006). The occurrence of different lineages in different host species and different places makes distinct parasite assemblages.

The spatial structure of haemoparasites has been characterized in a few migratory bird species, with mixed results. In South Africa, there was no geographic structuring of parasite communities in Red-billed Quelea *Quelea quelea*, but this host itself had similarly shown no phylogeographic structuring (Durrant et al. 2007). In North America, haematozoan parasites of Blackthroated Blue Warbler *Dendroica caerulescens* (Fallon et al. 2006) and Common Yellowthroat *Geothlypis trichas* (Pagenkopp et al. 2008) did not exhibit geographical structure. However, geographical structure was found in haemoparasite assemblages of House Finches *Carpodacus mexicanus* (Kimura et al. 2006) and American Redstarts *Setophaga ruticilla* (Durrant et al. 2008).

Many bird species have migratory divides, with one part of the population migrating in one direction and another part in another direction, usually with separate winter quarters for populations on each side of the migratory divide. These migratory divides may have originated between populations that spread from different glacial refugia, or formed in situ under local selection pressures (Møller and Szép 2011; Newton 2008). In Europe, many migratory species flying to Africa fly around important geographical barriers (the Alps, the Mediterranean Sea and the Sahara Desert), creating two main routes (Fig. 1): the Eastern flyway crosses the Bosphorus Straight or the Greek islands and follows the course of the Nile and the Red Sea shores, while the Western flyway runs along the Atlantic coast, entering Africa through Iberia and the Strait of Gibraltar. The Reed and the Great Reed Warblers (*Acrocephalus scirpaceus* and *A. arundinaceus*) are long distance migrant species that breed in reed beds all across Europe and winter in sub-Saharan Africa (Cramp 1992). They are known to migrate through both the eastern and the western European routes (Cramp 1992) and to have high migratory connectivity, previously proven by the analysis of feather isotopes (Yohannes et al. 2008; Procházka et al. 2009).

In this study, we investigated if the haemoparasite assemblages of Reed Warblers and Great Reed Warblers have any geographical structure in Europe and if such structure may be related to these birds' migratory movements. Both bird species have been extensively sampled for

haemoparasites in several European sites and are confirmedly infected by several parasite lineages [see all references concerning these two host species in the database of avian haemosporidians, MalAvi (Bensch et al. 2009)].

Methods

Field work

Sampling took place in four reed beds of Portugal (Fig. 1): Montemor-o-Velho (40º11' N, 8º41' W), Tornada (39º27' N, 9º03' W), Santo André (38°5' N, 8°48' W) and Vilamoura (37º05' N, 8º08' W). These sites cover a small latitudinal gradient of 370 km. Their still waters provide good breeding conditions for mosquitoes and other biting insects, potential haemosporidian vectors. These reed beds support important passerine communities, not only of resident birds such as the Cetti's Warbler (*Cettia cetti*), but also of several breeding migrants like the reed and the great reed warbler and of passage migrants such as the Willow Warbler (*Phylloscopus trochilus*). They are within the most south-western reed beds of Europe; therefore, they are among the last sites for the most western migrants to refuel before they cross the Mediterranean Sea on their way to Africa.

Reed Warblers and Great Reed Warblers were regularly captured with mist nets during their reproductive and migratory seasons (covering a period from March to October), from 2007 to 2009. Blood samples (around 40 µl) were collected with a 25 G or 30 G needle from their jugular or brachial veins and stored in 96% ethanol at room temperature. All birds were ringed, identified and

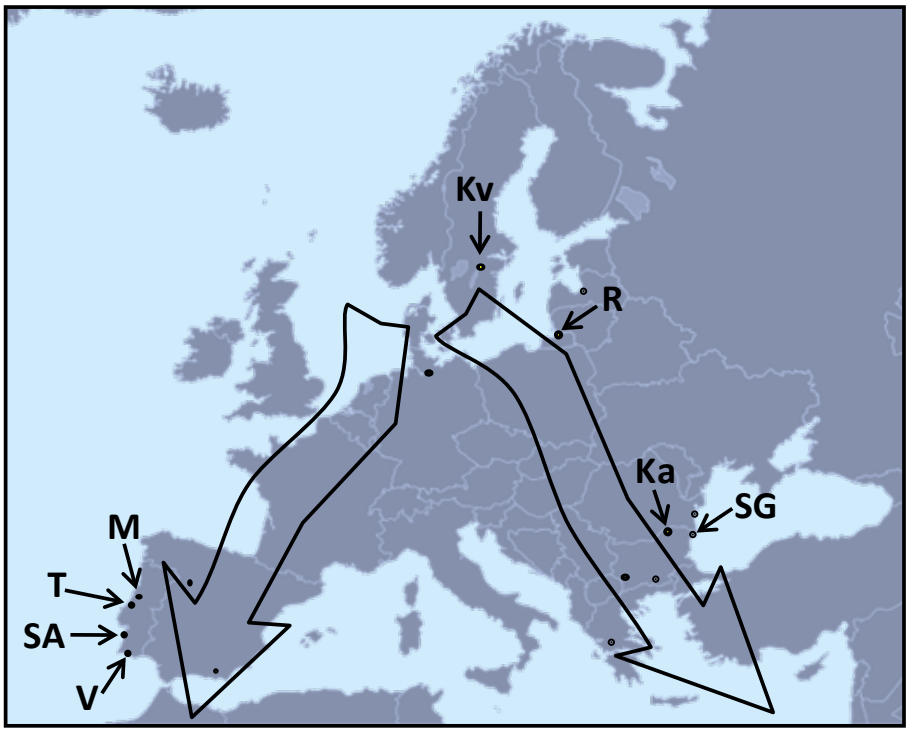

Fig. 1. Location of the European sites used to build the lineage pools of each host species (references in the MalAvi database, Bensch et al. 2009).

Eastern sites are: Kv = Kvismaren, R = Rybachy, Ka = Kalimok, SG = Sfântu Gheorghe. Western sites (Portuguese study sites) are: M = Montemor-o-Velho, T = Tornada, SA = Santo André and V = Vilamoura. Other black dots represent places for which small samples are available for one of the host species. Arrows symbolize the two main European migration flyways.

aged by plumage according to Svensson (1992) and released afterwards

.Laboratory work

Samples were diagnosed for infections using polymerase chain reactions (PCR) targeted at a portion of the parasite's cytochrome b gene. Variation in this genetic sequence defines parasite lineages, which may be considered as separate species (Bensch et al. 2004; Pérez-Tris et al. 2007).

After the DNA was extracted with ammonium acetate, the samples were tested for their good condition to undergo PCR by amplifying a fragment of the host's CDH gene, using the primers 0057F (CGTCAATTTCCATTTCAGGTA AG) and 002R (TTATTGATCCATCAAGTCTC). This reaction also informed about the sex of the individual. The resulting products were run in 2% agarose gels stained with ethidium bromide for band visualization under UV light.

A nested PCR (Waldenström et al. 2004) was used for infection diagnosis. A portion of the parasite's mitochondrial cytochrome *b* gene was amplified using the primers HaemNF/HaemNR2 for pre-amplification (Waldenström et al. 2004) followed by HaemF/HaemR2 (Bensch et al. 2000). Both primer pairs are specific to *Haemoproteus* and *Plasmodium* spp. (Waldenström et al. 2004). The final amplification products (479 bp) were run in a 2% agarose gel. Contaminations were controlled for by the inclusion of negative controls (water) during DNA extraction and during PCR (one blank for each set of 11 samples, plus one extraction control per each PCR plate). None of these controls ever showed amplification.

Samples that were negative for infection were confirmed by a second nested PCR (6.4% of negative samples turned positive after repetition), while all 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 genus and lineage. New parasite lineages and host-parasite associations were confirmed by repeating the whole process.

Statistical analysis

The four sampled Portuguese reed beds were considered as representative of the Western migration flyway. The parasite lineages infecting each bird species in each study site were listed down, constituting the site's parasite assemblage. At each site, the parasites of both host species were analysed separately; because both hosts share parasite lineages (Bensch et al. 2009), another analysis was done with both hosts pooled together. A research in the MalAvi database of avian haemosporidians (Bensch et al. 2009) provided a list of all parasite lineages discovered in these species in Europe, using the same molecular methods. Four sites were selected to represent the Eastern flyway (Fig. 1): Kvismaren, Sweden, 59°10' N, 15°25' E (Bensch et al. 2007; Hellgren et al. 2007b); Rybachy, Kaliningrad, Russia, 55°05' N, 20°44' E (Hellgren et al. 2007a; Hellgren et al. 2007b; Krizanauskiene et al. 2006); Kalimok, Bulgaria, 44°01' N, 26°26' E (Dimitrov et al. 2010; Zehtindjiev et al. 2008; Hellgren et al. 2007a; Hellgren et al. 2007b; Valkiūnas et al. 2008b); and Sfântu Gheorghe, Romania, 44º54' N, 29º36' E (Svoboda et al. 2009). These have 1844

Km maximum distance to each other and are, on average, 2670 Km away from the considered Western sites.

The sampling effort was not the same in each site, which is a frequent and unavoidable problem in comparative studies. Also, in some of the consulted articles, the percentage of adults in the bird sample is unknown. Because only the adults have already been to Africa, only they can carry African-transmitted lineages, hence providing information about the geographical structure of the parasite assemblages in the wintering grounds. However, this problem was lessened in this study because only parasite presence (instead of prevalence) was considered in the analysis of the assemblages, making it more independent from the sampling effort and from the sample's age structure.

The eight sites (both eastern and western) were compared two by two to calculate the similarity between the parasite assemblages of each possible pair of sites. The similarity between a site and each of the other sites was measured with the Sorensen similarity index (SSI):

$$SSI = 2C / (A+B)$$

where A and B are the number of parasites found in each of the two sites and C is the number of shared parasites between those two sites. This index varies between 0 (no similarity) and 1 (equality). To test if these similarity values were higher or lower than what could be expected at random in a European context, we compared these actual cases with the list of all parasite lineages reported for the same host species in Europe. Eight random parasite assemblages were simulated, each one corresponding to the host-parasite structure of one of the actual sites: we randomly chose parasite lineages from the European parasites' list for these hosts, until we reached the actual number of lineages observed in each host species and in each site (so the number of lineages per host in each site was kept constant). A thousand of these Monte Carlo simulations was made. The SSI's were calculated for these virtual assemblages grouped two by two, and the similarities between simulated assemblages were compared to the similarity values of the actual assemblages. All these calculations were done both for the parasites of each host and for the parasites of the two hosts considered together.

The effect of flyway and distance in site's similarity was assessed with an analysis of co-variance (ANCOVA), using the software STATISTICA 7.0 (Statsoft Inc. 2002), for each host species and for both species together. The dependent variable was the SSI of the site pairs, the covariate was the distance between the two sites and the categorical predictor was the flyway they belong to (with three categories: both eastern, both western or in different flyways). Because the data points are comparisons between two sites, they are not statistically independent (as changing the values of one site would change the comparisons between that site and all the others). Therefore, the correct ANCOVA's p-values were estimated using a Monte Carlo procedure: the values of the three variables were randomized 1000 times and ANCOVAs were performed on those simulated data. Statistically correct p-values were computed as the probability to find a randomly obtained F statistic that is equal or higher than the observed F.

Results

In the four Western study sites, a total of 421 reed warblers and 37 great reed warblers were sampled. The overall infection prevalence was of 54.1% for the reed warbler and of 24.7% for the great reed warbler. We recovered 15 lineages of haemoparasites from these hosts: six lineages of *Haemoproteus* sp. and nine of *Plasmodium* sp. (Table 1).

In the literature, we found records for 170 reed warblers and 962 great reed warblers sampled for avian malaria in the four sites representative of the Eastern flyway. In those sites, the overall prevalence was of 14.7% for the reed warbler and 47.7% for the great reed warbler. In the whole of Europe, a total of 32 parasite lineages (18 of *Haemoproteus* sp. and 14 of *Plasmodium* sp.) were recorded for these bird species (Table 1). From these 32 lineages, 14 were exclusively found in the Eastern flyway and 3 were exclusive of the western flyway; the remaining 15 were common to both flyways (13 of them could be found in these same host species and two are listed in MalAvi for different host species and/or different European sites). Seven of these lineages were shared between the two host species (Table 1).

When the parasites of both host species were pooled together, the Sorensen similarity index (SSI) for site pairs varied between 0.14 and 0.67 for two sites located in different flyways, 0.50 and 0.70 for pairs of western sites and 0.16 and 0.52 for eastern sites (Fig. 2). The results were similar when the two hosts were considered separately: the SSI for the Great Reed Warbler varied between 0.00 to 0.50 for sites in different flyways, 0.11 and 0.53 for eastern sites, and 0.00 and 0.50 for western sites. For the Reed Warbler, the SSI was between 0.00 and 0.60 for sites in different flyways, 0.25 and 0.50 for eastern sites and 0.4 and 0.82 for western sites.

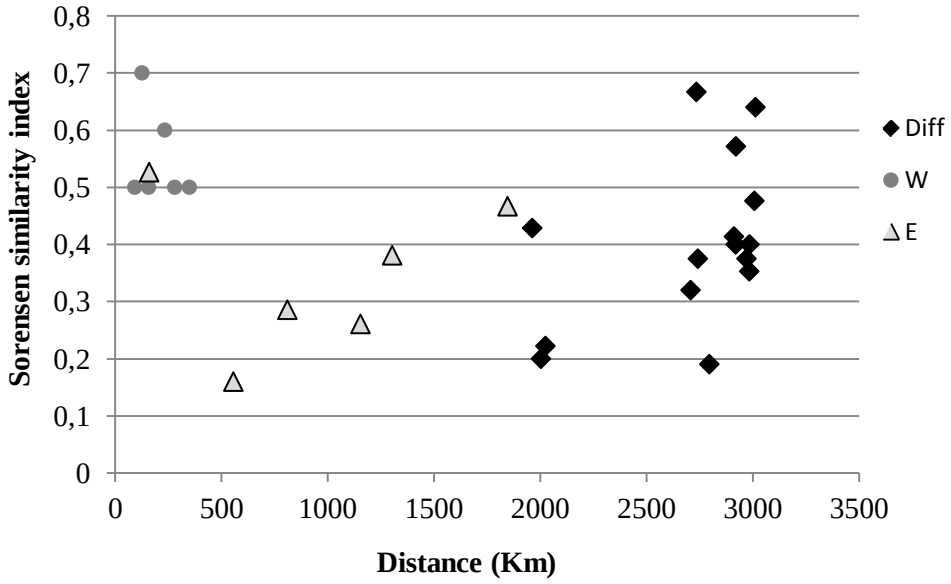

Fig. 2. Similarity (Sorensen Index) and distance in Km for all possible site pairs, according to the flyway the sites belong to: Diff = different flyway, W = both sites in the western flyway, E = both sites in the eastern flyway.

Table 1. Parasite lineages (genera *Haemoproteus* and *Plasmodium*) found in each host species and each study site, together with information on other records for those lineages in Europe.

Host species are: *A aru* = *A. arundinaceus*, *A sci* = *A. scirpaceus*. Eastern sites are: Kv = Kvismaren, R = Rybachy, Ka = Kalimok, SG = Sfântu Gheorghe. Western sites are: M = Montemor-o-Velho, T = Tornada, SA = Santo André and V = Vilamoura. O = other site located in that flyway.

Genus	**Lineage**	**Eastern flyway**		**Western flyway**		**Only one flyway?**	**Different flyway, in other hosts?**
		A aru	***A sci***	***A aru***	***A sci***		
Haem	ACSTE1	Ka SG				**Yes, E**	No
	ARW1		R Ka SG		O	No	
	GRW01	Kv R Ka O		M T SA		No	
	GRW03	Ka				**Yes, E**	No
	GRW05	Kv Ka SG				**Yes, E**	No
	GRW08	Kv				**Yes, E**	No
	GRW13	Kv				**Yes, E**	No
	GRW16			SA		**Yes, W**	No
	HIPOL1				V	**Yes, W**	No
	MW1		R Ka		M T SA V O	No	
	PHSIB1	Kv				**Yes, E**	No
	RW1		R		M T SA V O	No	
	RW2	Kv				**Yes, E**	No
	RW3		R			**Yes, E**	No
	SW1		Ka		SA	No	
	TURDUS2		R			Yes, E	**Yes:** *Cyanistes caeruleus*, UK; *Turdus merula*, France.
	WW1		R			Yes, E	**Yes:** *Phylloscopus trochilus Cyanistes caeruleus*, UK.
	WW2	Kv				**Yes, E**	No
Plasm	GRW02	Kv Ka O		M		No	
	GRW04	Kv Ka SG		M SA V	M SA V	No	
	GRW06	Kv Ka			M SA V O	No	
	GRW07	Kv				**Yes, E**	No
	GRW09	Kv				**Yes, E**	No
	GRW10	Kv				**Yes, E**	No
	GRW11	Ka			M SA	No	
	GRW14	Kv				**Yes, E**	No
	GRW15	Kv				**Yes, E**	No
	GRW17			SA		**Yes, W**	No
	RTSR1	Kv Ka			SA V	No	
	SGS1	Kv Ka	R	SA	T SA V	No	
	SW2	SG			M	No	
	SW5	Kv			SA V	No	

Table 2. ANCOVA results: influence of the variables distance (co-variate) and flyway (factor) in the Sorensen similarity index (SSI) of a site pair.
SSI were calculated for the parasites of each host species and for both host species pooled together.

Host(s)	Variable	SS	d.f.	MS	F	p	Correct p
Great Reed	Distance	0.168	1	0.168	7.456	0.014	0.010
Warbler	Flyway	0.413	2	0.207	9.155	0.002	< 0.01
Reed	Distance	0.028	1	0.028	0.614	0.441	0.369
Warbler	Flyway	0.025	2	0.013	0.276	0.761	0.699
Both hosts	Distance	0.068	1	0.068	3.917	0.059	0.040
	Flyway	0.200	2	0.010	5.781	0.009	0.003

Simulations showed that any two sites situated in the same flyway were not more similar than random, neither for the parasites of both hosts pooled together ($p = 0.130$ for the eastern flyway, $p = 0.065$ for the western flyway) nor for the Reed Warbler only ($p = 0.062$ for eastern sites and $p = 0.347$ for western sites). However, parasite assemblages were more similar than random in both flyways for the Great Reed Warbler ($p = 0.02$ in the east and $p = 0.009$ in the west).

In the ANCOVA, for the two hosts together as well as for the Reed Warbler, both the distance between sites and the flyway had a significant effect on the similarity between two sites, once the p-level was corrected for the data's lack of independence. For the Great Reed Warbler, none of these variables contributed significantly to explain site similarity (Table 2).

Discussion

The fact that some parasite lineages were exclusive of one of the flyways suggests that the haemoparasite distribution has some geographic structure across Europe. This is confirmed by the significant influence of the variable flyway in the similarity between sites' assemblages, when both hosts are considered and when only the Reed Warbler is analysed. However, in those two cases, neither the eastern nor the western assemblages were more similar to each other than random, indicating that the observed geographical differences do not correspond to a strong geographical structure of the parasite community. The much greater number of lineages found in the Eastern flyway probably reflects the larger sample size of Great Reed Warbler in the eastern locations, because a larger sampling effort increases the probability to find rare lineages. The huge sample size in the location of Kvismaren (782 Great Reed Warblers; Bensch et al. 2000) was for the most part responsible for the finding of this host's rare lineages, as many of those parasites (10) were not found anywhere else. This expanded the list of lineages that entered the simulations of random assemblages, making the similarity

between actual sites turn significant for this host species.

The shorter average distance between the studied western sites than the eastern sites may contribute to the higher similarity between western sites; this is confirmed by the fact that not only the flyway but also the distance between sites significantly affected site similarity. Therefore, the observed spatial differences should not be created primarily by the hosts' migratory movements. For the Great Reed Warbler, neither distance between sites not the flyway they belong to affected site similarity, confirming the lack of spatial structure of this host's parasite assemblages.

In host species with low migratory connectivity, a weak geographical structure of haemoparasites could reflect the mixing of different host populations, either in their breeding or wintering grounds. However, this should not occur in a large scale in the two studied host species, both of which showed high migratory connectivity in isotope studies (Procházka et al. 2009; Yohannes et al. 2008). Therefore, parasite lineages should be homogenized by other processes, such as: 1) these hosts' natal dispersal on the breeding grounds, given that these grounds form a continuum through Europe; or by 2) movements of other possible host species – always associated with the presence of competent vectors (Møller and Szép 2011). Both in Europe and in Africa, host-generalist lineages may use several different avian species (including resident ones) to colonize different sites, provided they find suitable vectors in those new locations. In this way, transmission corridors should be created that may ultimately connect haemosporidians from the warbler populations of the two main flyways, contributing to homogenize the parasite assemblages of the studied avian species. The same homogenization process has been suggested for *Leucocytozoon* sp. parasitizing resident passerines in Europe, wherein parasites showed no geographical structure and dispersed independently of their hosts (Jenkins and Owens 2011). Some of the lineages analysed in the present study, such as SGS1 and GRW04, fit into this description because they are host-generalists with known alternative hosts (Hellgren et al. 2009). For host-specialist lineages such as GRW05 (with no other hosts known to date), colonization of new sites independently of host movements should be reduced; indeed, GRW05 appeared only in the eastern flyway. The specialist lineages may be the ones that confer some structure to parasite distribution, causing the flyway to have the observed significant effect in site similarity.

The theory that migratory connectivity is advantageous to birds because it reduces the chances of encountering novel parasites and contributes to lower parasite loads (Møller and Szép 2011) assumes that the parasite communities are spatially structured. This is not what was found for haemosporidians in this and other studies (Jenkins and Owens 2011; Fallon et al. 2006; Pagenkopp et al. 2008). If the haemoparasite assemblages are not significantly different between distinct routes, then breaking connectivity does not imply that the hosts will find unknown parasites. Therefore, haemoparasite avoidance can hardly cause a selective pressure strong enough to lead to the evolution of connectivity or to the maintenance of connectivity that originated when host populations spread from different refugia. This theory of parasite avoidance may apply to other

kinds of parasites, as long as they show geographical structure, are host-specialist and tightly coevolved with their hosts (Møller and Szép 2011).

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CHAPTER 4

No evidence of release from haemoparasites in introduced wetland passerines

Rita Ventim, Luisa Mendes, Jaime A. Ramos, Helder Cardoso and Javier Pérez-Tris

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Two birds of introduced species, a Waxbill in Santo André (left) and a Black-headed Weaver in Vilamoura (right).
Photos by: Rita Ventim (2007)

No evidence of release from haemoparasites in introduced wetland passerines

Abstract

The Enemy Release Hypothesis states that the enhanced performance of a species introduced to a new area can be due to the absence of its natural co-evolved enemies such as parasites, pathogens or predators. In order to evaluate this hypothesis, four introduced and six local passerine species were tested for haemosporidioses (genera *Haemoproteus* and *Plasmodium*) in four Portuguese reed beds. The exotic bird species harboured less infecting lineages and at a lower prevalence than the native species but, when phylogeny was controlled for, there were no significant differences between exotics and natives. Therefore, we found no evidence that the Enemy Release Hypothesis applied to the haemoparasites of these exotic bird species. Two local *Plasmodium* lineages infected the exotic species: one of them (SGS1) was the most generalist and prevalent lineage in the native species, so it could be expected to be present in the exotics at random. The other lineage (PADOM01) was rarer in the sampled community, but was present in native hosts that are phylogenetically close to the infected exotic species. Therefore, the colonization of the exotic species by haemoparasites seems to be phylogenetically constrained or regulated by random processes.

Keywords: *Plasmodium, Haemoproteus*, haemosporidioses, avian malaria parasites, enemy release hypothesis, introduced birds, exotic species.

Introduction

Most of the exotic species that are introduced to new areas never become naturalized, even if they find suitable environmental conditions (Duncan et al. 2003). Yet some manage to establish self-sustained populations in the area of introduction, increase in numbers and spread, sometimes achieving a higher growth rate or survivorship than they originally had (Torchin et al. 2001). The exotic species that are so successful as to become invasive may unbalance the ecosystems and drive losses in the biodiversity of native species' populations; they are a major cause of extinction at present times (Vitousek et al. 1997; Mack et al. 2000).

The Enemy Release Hypothesis (ERH) states that a species introduced to a new area may have an enhanced performance due to the absence of its natural co-evolved enemies such as parasites, pathogens or predators (Torchin et al. 2001; 2003). In particular, the release from its original parasites can give the introduced species an advantage over their competitors in the new area (Torchin et al. 2001). The original parasites are often lost before or during their hosts' introduction (Torchin et al.

2003; MacLeod et al. 2010). Even if the parasites arrive in the founder hosts' population, they can fail to colonize the new region for three reasons: 1) propagule pressure: too few parasites or too few infected hosts may have been introduced (MacLeod et al. 2010); 2) absence of competent or vectors in the new region, in the case of vector-transmitted parasites (Torchin et al. 2003); and 3) transmission rates can be too low to sustain the parasite's population (Anderson and May 1978; MacLeod et al. 2010).

Upon arrival, an introduced host will also be exposed to the local parasites of the new area. However, these parasites have not co-evolved with the introduced host, so most of them will not be able to infect the introduced species. Therefore, the ERH postulates that the colonization by new parasites should not make up for the loss of the original parasites (Torchin et al. 2003; Marzal et al. 2011).

Avian haemosporidians of the genera *Haemoproteus* and *Plasmodium* are an interesting system for testing the ERH, because they have a broad geographical distribution and infect bird species of a wide range of families (Waldenström et al. 2002; Valkiūnas 2005). Different haemoparasite species and lineages infect distinct host species and have different host-specificity (Hellgren et al. 2007b). Due to the potential deleterious effect of these parasites on host health and reproduction (Merino et al. 2000b; Marzal et al. 2005; Norte et al. 2009; Knowles et al. 2010), an introduced bird species could gain a fitness advantage when they escape from haemoparasites.

In Portugal, one of the most successful introduced avian species is the Waxbill *Estrilda astrild* (family Estrildidae, superfamily Passeroidea). This species is originally from Sub-Saharan Africa, was released in coastal Portugal in the 1960s and has quickly spread to most of continental Portugal and part of Spain (Silva et al. 2002). The Red Avadavat *Amandava amandava* (Estrildidae: Passeroidea), from tropical South Asia, was introduced in Italy in the 1980s and has also colonized Spain and Portugal (Matias 2002). The Black-headed Weaver *Ploceus melanocephalus* and the Yellow-crowned Bishop *Euplectes afer* (Ploceidae: Passeroidea) were introduced from Africa in the 1980s. All these species arrived to Portugal as accidental escapes from the pet trade, and have successfully colonized wetlands and riverine areas (Matias 2002; Matias et al. 2007). This study compares the haemosporidian infections of these exotic passerines with those of native species, to test if the ERH applies to the introduced species. To our best knowledge, it is the first time that haemosporidians from these exotic bird species were investigated.

Methods

Field work

Birds were captured using mist nests from March 2007 to September 2009 in four coastal wetlands of Portugal, where nesting of exotic species was previously confirmed: Paul do Taipal (40º11'N 8º41'W), Paul de Tornada (39°26'N 9°08'W), Lagoa de Santo André (38°4'N 8°48'W) and Vilamoura (37º04'N 8º07'W). All these wetlands provide good conditions for the development of mosquitoes and other biting insects, potential haemosporidian vectors. Its

reedbeds are important stop-over, wintering and nesting sites for migrants (such as the Reed and Great Reed Warblers, *Acrocephalus arundinaceus* and *A. scirpaceus*) and resident birds (such as the Cetti's Warbler *Cettia cetti*).

Four exotic species were found, all belonging to the superfamily Passeroidea: the Black-headed Weaver and the Common Waxbill were present in great numbers and could be sampled during the three years, while only punctual samples could be taken for the Yellow-crowned Bishop and the Red Avadavat. Among the native passerines, the six most abundant species in the study areas were sampled: the Reed and the Great Reed Warblers, the Cetti's Warbler, the Savi's Warbler *Locustella luscinioides* (superfamily Sylvioidea), the House Sparrow *Passer domesticus* and the Tree Sparrow *Passer montanus* (superfamily Passeroidea). After the birds were ringed, a blood sample (around 40 µl) was collected from their jugular or brachial vein using a 25 G or 30 G needle and stored in 96% ethanol.

Molecular analysis

DNA was extracted from the blood samples by a standard ammonium acetate protocol. To confirm the good condition of the extracted DNA, all samples were tested using a universal bird sexing protocol that amplifies a CDH gene's fragment by polymerase chain reaction (PCR), using the primers 0057F (3' CGTCAATTTCCATTTCAGGTAAG 5') and 002R (3' TTATTGATCCATCAAGTCTC 5'). The reaction products were run in 2% agarose gels for band visualization; the appearance of one or two bands confirmed the successful DNA amplification.

The samples were diagnosed for infections by amplification of a portion of the parasite's cytochrome b gene. Variation in this genetic sequence defines parasite lineages, which may be considered as separate species (Bensch et al. 2000; Pérez-Tris et al. 2007). We used a nested PCR protocol (Waldenström et al. 2004) with primers that are specific for the genera *Haemoproteus* and *Plasmodium*: HaemNF/HaemNR2 (Waldenström et al. 2004) for the pre-amplification PCR, followed by HaemF/HaemR2 (Bensch et al. 2000) for the specific PCR. 1 µl of the products of the pre-amplification PCR was used as template for the second PCR. False positives (contaminations) were controlled for by including a negative control per each 24 samples during extraction and a negative control (water) for each 8 samples during PCR. None of these controls ever showed amplification.

Samples that were negative for infection were confirmed by a second nested PCR, while all 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. New parasite lineages and host-parasite associations were confirmed by repeating the whole process.

Statistical analysis

The Red Avadavat and the Yellow-crowned Bishop were excluded from these analyses because their sample size was too small to be representative of the species' prevalence and number of infecting

lineages. All other sampled bird species were classified according to their host type (as exotic or native) and the effect of this binary variable on prevalence and on the number of parasite lineages per species was tested performing two ANOVAs on STATISTICA 7 (Statsoft Inc. 2002). The obtained values for the F statistics were then used to perform a phylogenetic ANOVA using the ''Phenotypic Diversity Analysis Programs'', PDAP (Garland et al. 1993).

In a conventional ANOVA, the obtained value of the F statistics is compared with the standard distribution of F, with degrees of freedom determined by the number of groups being compared and the total number of observations (in this case, species) in the dataset. However, the phylogenetic relationships between species prevent them from being statistically independent data points and make it difficult to know how many degrees of freedom should be considered (Garland et al. 1993). Therefore, the obtained F values cannot be directly compared with the conventional tabulated distributions of the F statistics to find the associated probability value and significance. Phylogenetically correct significance values must be obtained from empirical null distributions of F statistics, which can be created using computer simulation models of traits evolving along known phylogenetic trees (Garland et al. 1993).

The phylogenetic tree of the sampled species was build following Johansson et al. (2008), Treplin et al. (2008) and Arnaiz-Villena et al. (2009). Using PDSIMUL (Garland et al. 1993), 1,000 sets of tip values were simulated for the two traits under study (infection prevalence and number of lineages per host species), assuming a gradual Brownian motion model of evolutionary change. Prevalence was bounded to vary between zero and one, while the number of lineages was bounded between zero and 30 (the highest number registered for a passerine host in the MalAvi database; Bensch et al. 2009). We used the between-species means of real data as both starting values and expected means of simulated tip values. The expected variances of the simulated tip data were set equal to the variances of the real data. The program PDANOVA (Garland et al. 1993) calculated the F values for the two traits in the 1,000 simulations, generating the empirical null distributions of F. The upper 95% percentile of these distributions, calculated with STATISTICA 7, was the critical value against which the F-ratios of our real dataset were compared (Garland et al. 1993).

Results

A total of 1120 individual birds were sampled, from which 945 belonged to native species and 175 were exotic (Table 1). Native species had total infection prevalences ranging from 15% to 56% of infected individuals (Table 1), with a mean of 37.6%. In the exotic species, prevalences were of 1% in the Common Waxbill and 8% in the Black-headed Weaver. The host type (exotic/native) seemed to have a significant effect on the infection prevalence by species using a conventional ANOVA ($F = 6.74$, $df = 1$, $p = 0.031$). However, this F-ratio was not higher than the critical value of the empirical null distribution ($F = 9.64$), so this effect was not significant in the phylogenetic ANOVA. This discrepancy may be explained by the fact that species' status (exotic or native) has

an important phylogenetic component in our study, in which all exotics belong to the same superfamily.

All the native species were found to be infected by at least one lineage of *Plasmodium*, and four of them were also infected by at least one *Haemoproteus* lineage (Table 1). The *Plasmodium* SGS1 was the lineage infecting a larger number of native host species. Among the exotic species, no infection was found in the Red Avadavat, while the other three species were infected by one *Plasmodium* lineage each (Table 1). No *Haemoproteus* parasites were found in the exotic species.

On average, each exotic host species was infected by fewer lineages than a native host species (Table 1), but this effect was not statistically significant either in a conventional ANOVA ($F = 2.85$, $df = 1$, $p = 0.142$) or in the phylogenetic ANOVA (critical value of the

Table 1. Sample size, number of infections and number and name of the parasite lineages found per bird species.

Bird superfamily, in brackets, is: P=Passeroidea, S=Sylvioidea. Prevalence, in brackets, is given as a percentage. Lineage names marked ^{H} belong to the genus *Haemoproteus*, unmarked ones are *Plasmodium* lineages.

Host type	Bird species (Superfamily)	n	Nr. Infected (Prevalence)	Nr. of lineages	Lineage names
Exotic	Red Avadavat (P)	4	0	0	-
	Common Waxbill (P)	104	1 (0.96%)	1	SGS1.
	Yellow-crowned Bishop (P)	2	1	1	PADOM01.
	Black-headed Weaver (P)	65	5 (7.7%)	1	SGS1.
Native	House Sparrow (P)	121	45 (37.2%)	4	PADOM01, ^{H}PADOM23, GRW11, SGS1.
	Tree Sparrow (P)	53	19 (35.8%)	1	SGS1.
	Great Reed Warbler (S)	37	20 (54.1%)	6	^{H}GRW01, GRW02, GRW04, ^{H}GRW16, GRW17, SGS1.
	Reed Warbler (S)	410	103 (25.1%)	11	GRW04, GRW06, GRW11, ^{H}HIPOL01, ^{H}MW1, ^{H}RW1, ^{H}SW1, RTSR1, SGS1, SW2, SW5.
	Savi's Warbler (S)	46	7 (15.2%)	5	COLL1, GRW04, GRW06, ^{H}MW1, WW4.
	Cetti's Warbler (S)	305	171 (56.1%)	4	CET01, GRW11, SGS1, SYAT05.

empirical null distribution = 8.81, much higher than the real case's F-ratio).

All parasite lineages that were found in the exotic species were also present in the native species. The *Plasmodium* SGS1, found in the Waxbill and the Black-headed Weaver, was the most prevalent lineage in the native species (causing 61.9% of all infections in native individuals) and infected 5 of the 6 sampled native species. PADOM01, found in one Yellow-crowned Bishop, also infected the House Sparrow; but it was present only in 4.5% of the infected individuals of that species. Moreover, in the set of native species, PADOM01 accounted for only 0.55% of all infections.

Discussion

The haemosporidian infections of these exotic species at their original home range have not yet been thoroughly studied. However, three *Plasmodium* lineages were already found for the Red Avadavat in India (Ishtiaq et al. 2007) and one was found for the Waxbill in Tanzania (Beadell et al. 2006); none of those lineages were present in the Portuguese study sites. Although nothing is known about the original infections of Yellow-crowned Bishops and Black-headed Weavers, there are two reasons to believe that these species are also infected by some haemosporidian lineages in their original home range: 1) the genera *Haemoproteus* and *Plasmodium* have a wide distribution in the majority of the sampled bird species around the globe (Valkiūnas 2005); and 2) several other species of the Estrildid and Ploceid families were found to be infected by lineages of both haemoparasite genera (Beadell et al. 2006; Hellgren et al. 2007b; Durrant et al. 2007). The original haemosporidian infections of these exotic species were probably eliminated from the studied populations (Lima et al. 2010; Marzal et al. 2011), either because they were absent in the arriving individuals (by founder effect; MacLeod et al. 2010) or because they were lost after arrival (by propagule pressure, too low transmission rates or the absence of competent vectors; (Torchin et al. 2003; MacLeod et al. 2010).

From the lineages that infected the exotic species at the study sites, PADOM01 was never found in the original home range of the exotic species, while SGS1 was found in Sub-Saharan Africa (Hellgren et al. 2007b), but not in Ploceids or Estrildids (although some species of those families were sampled). Therefore, it is very likely that these lineages were acquired locally. This is supported by the fact that these lineages were also found in the native bird species at these sites.

One of the predictions of the enemy release hypothesis (ERH) is that exotic species should be less affected by natural enemies at the introduction sites than their native competitors (Torchin et al. 2003; Torchin and Mitchell 2004; Lima et al. 2010). Our study found no evidence that this was true for haemoparasites in the study area, because the differences between the two host types in prevalence and in number of lineages were not significant once the results were corrected for the hosts' phylogeny. At the time of this study, the Red Avadavat, the Black-headed Weaver and the Yellow-crowned Bishop were infected with some of the local haemoparasites. With the present data, there is no way to know if these parasites were able to infect the exotic as soon as they were present in

the study areas. If, on the contrary, the local parasite colonization of the exotic species took some time, then there could have been a period of release from parasites for these exotic species, from their introduction into the study areas to the time when the local lineages could infect them; this would have given the introduced species a temporary competitive advantage over the native species, which could contribute to explain the Waxbill and the Black-headed Weaver's success in colonizing the study area (Lima et al. 2010). However, even if this moment of parasite release occurred, it does not seem to continue at the present time. Future studies would be needed to understand if the colonization process of the introduced hosts will progress to include other local lineages and raise the prevalences in the exotic species.

From the local lineages that could have colonized the exotic hosts, SGS1 was the most expected because this is the most abundant parasite lineage that was sampled in the study area, and also the most host-generalist. Indeed, it is one of the most generalist lineages worldwide, infecting a great number of birds of different orders (Bensch et al. 2009 and references therein). The most host generalist parasites can also be the most prevalent in single host species and in a community (Hellgren et al. 2009). Because of its abundance, SGS1 is very likely to be transmitted to the exotic hosts by chance; and, because of its generality, it was expected to be able to adapt to these new hosts and succeed in infecting them.

However, PADOM01 is a rare lineage in the studied native community (causing only 0.55% of all infections), so its presence in the Yellow-crowned Bishop is not easily explained by random processes. In previous studies, this lineage has been found in few host species, all from the superfamily Passeroidea (Johansson et al. 2008): the House Sparrow (Bonneaud et al. 2006; Dimitrov et al. 2010), the Spanish Sparrow *Passer hispaniolensis* (Marzal et al. 2011) and the Yellow Wagtail *Motacilla flava* (Hellgren et al. 2007b). In all these cases, this lineage's prevalence in the host was similar to that of the present study. Therefore, globally, this parasite lineage seems to be more host specialist than SGS1, only being able to infect a group of closely related hosts. Its presence in the Yellow-crowned Bishop can be explained by its special affinity to Passeroids. A larger sample of Yellow-crowned Bishops would be needed to know if this species also hosts other parasite lineages.

In summary, in this community of reedbed passerines, we could not find evidence that the exotic species were under a release from haemoparasites, although the exotic species seemed to have lost parasites of their original home range. Some of the local parasite lineages were able to colonize these new available hosts, and such colonization seems to be phylogenetically constrained. However, all available exotic species in this study were phylogenetically related and had a similar establishment success; the haemoparasite scenario may be different for other introduced bird species, with different phylogenies and colonization processes. Further research is needed to understand whether parasitism may play a role in deciding the settlement success of introduced passerines in general.

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CHAPTER 5

Avian malaria infections in western European mosquitoes

Rita Ventim, Hugo Osório, Ricardo Lopes, Jaime A. Ramos, Javier Pérez-Tris and Luísa Mendes

A CDC light trap hanging from a tree in Tornada, with a bag of CO_2 next to it and a cage above it.
Photo by: Rita Ventim (2009).

Avian malaria infections in western European mosquitoes

Abstract

Avian malaria parasites (*Plasmodium* sp.) have a complex life cycle with mosquitoes and birds as hosts. Yet, we still have a poor understanding of the vector-parasite relationships. This study described the community of potential avian malaria vectors in four Portuguese reedbeds, tested if their geographical distribution differed and investigated their *Plasmodium* infections. In one of these sites, the mosquitoes' feeding preferences were evaluated using CO_2, a mouse and a bird as baits. The most abundant species were *Culex pipiens*, *Cx. theileri* and *Ochlerotatus caspius*. *Coquillettidia richiardii* was also very abundant at one site, where *Cx. theileri* and *Oc. caspius* were absent. Two avian *Plasmodium* lineages, SGS1 and SYAT05, were found in unengorged *Cx. pipiens* and *Cx. theileri* (respectively), suggesting these mosquitoes were competent vectors of those lineages. These 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* appeared to be an opportunistic feeder, while *Cx. pipiens* appeared to be mammophilic, perhaps because the used avian bait was not its preferential target. To our knowledge, this is the first report of detection of avian *Plasmodium* DNA from European mosquitoes.

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; Valkiūnas 2005; Njabo et al. 2009). A mosquito becomes infected by feeding on 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 (Valkiūnas 2005; Atkinson and van Riper III 1991). 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. Every *Plasmodium* species can 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, reedbeds 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 (Sol et al. 2000). This study characterized the mosquito community of four Portuguese reedbeds 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.

Methods

Study area:

This study took place in four Portuguese marshes: Taipal (40º11'N, 8º41'W), Tornada (39°26'N, 9°08'W), Santo André (38°4'N, 8°48'W) and Vilamoura (37º04'N, 8º07'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 (pers. observation). The vegetation is dominated by vast extensions of reed (*Phragmites australis*) which harbour many bird species of reedbed 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 refuelling 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. in press-a).

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 with CDC (Centre for Disease Control) light traps with UV light (John W. Hock Company, Gainesville, FL, USA), baited with CO_2 (dry ice) and located on the marshes' shores. 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. in press-a; in press-b).

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 abundance surveys. Apart from previously referred the 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 to 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.

All the collected insects were inactivated by refrigerating them at 4ºC and then kept frozen at -20ºC until they were analysed.

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 -20ºC 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 analysed 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 (COI). 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 100µM dNTPs (deoxynucleoside triphosphates), 0.75 µl of AmpliTaq DNA polymerase (Applied Biosystems, Foster City, California), 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 94º C, followed by 35 cycles of 94º C for 30 sec, 45º C for 30 sec, 71º C for 60 sec, and ended with final a extension step at 72º 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 analysed for the identity and infection status of the blood donor. 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 1.4µl DNA, 0.5µl of each primer (10 µm), 5µl Phusion® Flash High-Fidelity PCR Master Mix (Thermo Fisher Scientific, USA) and 2.6µl of dH2O. It included one cycle of 30s at 98 °C, 38 cycles (3s at 98°C, 10s at 59°C, and 12s at 72°C), and a final extension at 72 °C for 5 min. The results were verified through electroforesis 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.

Haemoparasite 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. in press-b). 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 at10 mM and 0.1 µl of DNA polymerase at 5 Ud/µl. The thermal profile had an initial denaturation step at 94 ºC for 3 min, followed by cycles of 94º C for 30 sec, 50º C for 30 sec, 72º C for 45 sec, and a final elongation step at 72º C for 10 min. The pre-amplification 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, 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. (in press-b). The use of juvenile birds guarantees that all infections were transmitted locally (Ventim et al. in press-a; 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 sub maximal 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 (%) is:

MIR = 100 × number of PCR positive samples / total number of analyzed mosquitoes.

The feeding preference of each mosquito species from Tornada was analyzed comparing the number of mosquitoes captured with each bait (CO_2, House Mouse and Bengalese Finch), using the non-parametric Kruskal-Wallis analysis of variance (Statsoft 2011).

Results

Mosquito abundances

The traps baited only with CO_2 collected 2702 unengorged female mosquitoes belonging to 13 species (Table 1). Only one set of mosquitoes could not be identified to the species level: that belonging to the species complexes *Anopheles claviger* s.l. and *Ochlerotatus detritus* s.l.. These two species complexes contain more than one species in continental 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 *An. atroparvus* (from the *An. maculipennis* s.l. complex), *Culex perexiguus* (belonging to *Cx. univittatus* s.l.) and *Cx. 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, 2038 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. 1, a-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 (χ^2_9 = 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 than expected in Taipal (and *Cx. pipiens* also in Vilamoura), while *Oc. caspius* was more abundant in Santo André and *Cq. richiardii* was more abundant than expected in Tornada. In the local communities of juvenile birds (Ventim et al. in press-b), there were significant differences in overall malaria prevalence between sites (χ^2_3 =

10.63, p = 0.014); these should be due to the differences in the most abundant lineage, SGS1 (χ^2_3 = 9.72, p = 0.021), which is more prevalent than expected in Taipal and Santo André. 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 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. theilei* (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	1153
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	1220	247	664	2702
Nr. Species	9	11	7	6	13

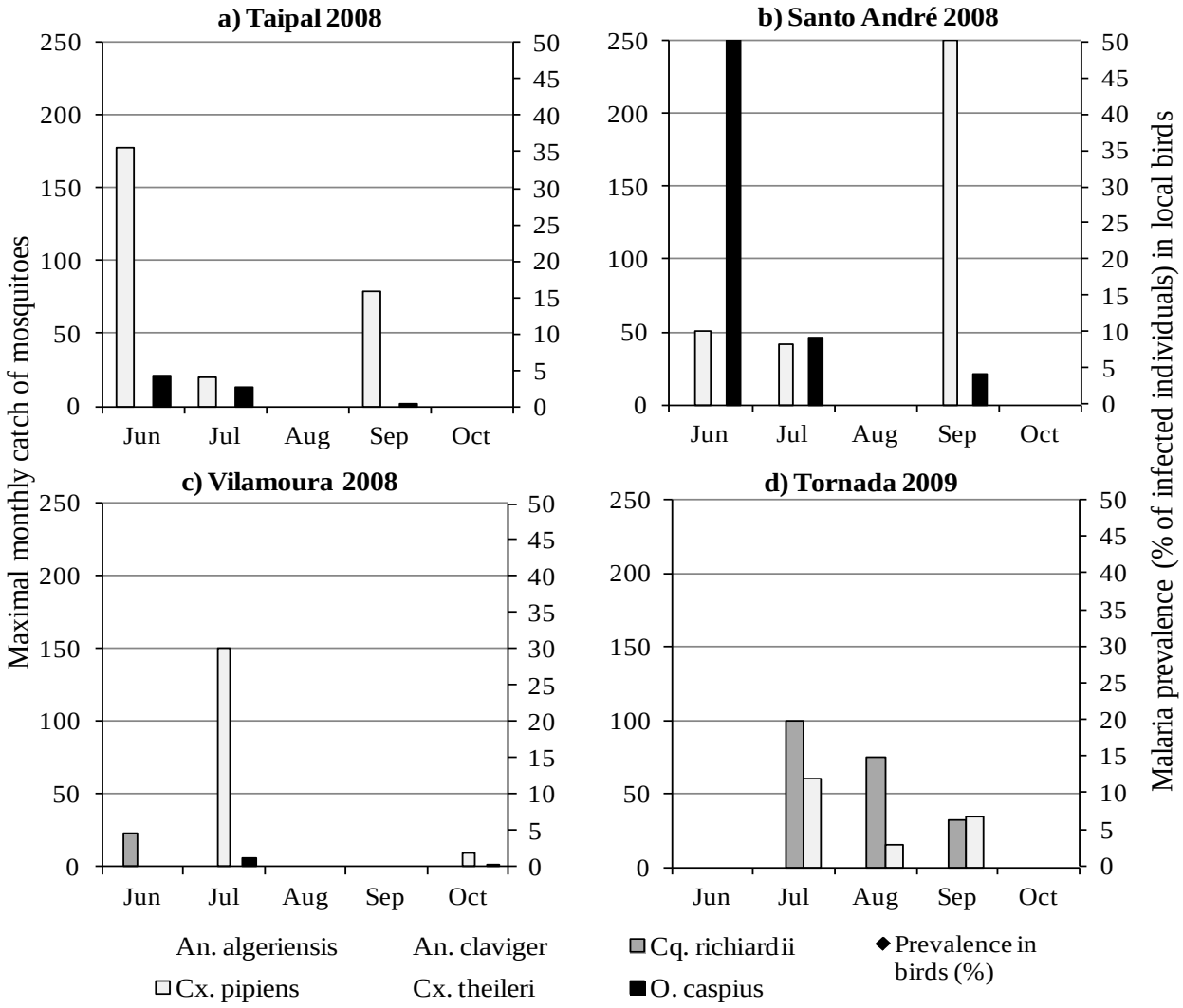

Figure 1. Monthly abundances of mosquitoes (bars are the species that comprise more than 1% of the site's captures) and monthly malaria prevalence in juvenile birds per site (black diamonds).
Left axes measure mosquito abundance (maximum catch per trap), right axes measure overall malaria prevalence (% of infected individuals) in local 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 *An. 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 *Cs. annulata* (52%) and *Cx. pipiens* (51%). *Cq. richiardii* was more attracted to the CO_2-baited traps than to any animal bait, thus behaving as an opportunistic feeder (H(2;24) = 4.60, p = 0.10).

Forty engorged females were captured in Tornada (Table 2) and it was possible to identify the blood meal's source 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 species that roosts in great numbers inside Tornada's reedbed. In two blood meals of *Cq. richiardii*, a human donor was recognized; however, human contamination of the samples was not ruled out.

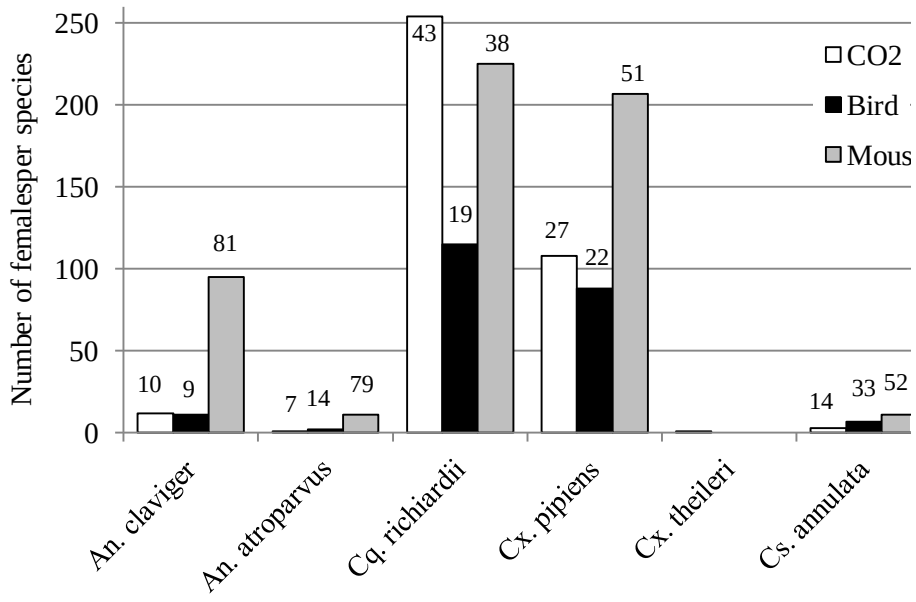

Fig. 2. Mosquito captures (number of females per species) using CO2 (white bars) and two different animal baits, a bird (Lonchura domestica, in black) or a mouse (Mus musculus, in grey). Numbers on the bars represent the percentage of mosquitoes attracted to each of the baits. Catches took place at Tornada, on eight nights in August and September 2009.species is shown above each column.

Table 2. Analysis of blood meals of engorged females – number of identified meals and identity of the blood donors.

Mosquito species	**Id. 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)

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* (Osório et al. 2010; Almeida et al. 2008). Tornada seemed to be different because the most abundant mosquito was *Cq. richiardii*, a relatively rare species in Portugal (Osório et al. 2010; Almeida et al. 2008), but 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. in press-b) 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 morpho-species *P. 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. 2009); and *Cx. theileri* has been described as a vector of the morpho-species *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 predictable because the majority of the detected bird infections must belong to the chronic phase of infection (Ventim et al. in press-b; 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. 2009; Valkiūnas 2005). These two lineages were also present in the passerine communities of the study sites: SGS1was present at the four sites, in Cetti's Warbler (*Cettia cetti*) and in six other resident, migrant and exotic passerine species;

SYAT05 was found in Cetti's Warbler in three of the four sites (Ventim et al. in press-a). Both lineages must be transmitted locally, given that they were both present in the resident Cetti's Warbler, a species which is known to spend most of its time in the reedbed ((Cramp 1992); Cardoso, pers. observation).

In our study sites, SGS1 was the most prevalent lineage in the studied bird communities (17% overall) (Ventim et al. in press-a), 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. 2007a; 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. (in press-a) 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.

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 *An. maculipennis* s.l. (the species group that includes *An. atroparvus*) was described as mammophilic (Ponçon et al. 2007; Balenghien et al. 2006) and *Cq. richiardii* as an opportunistic feeder (Balenghien et al. 2006), *Cx. pipiens* is usually referred to as primarily ornithophilic (Savage et al. 2007; Apperson et al. 2004), unlike what was found in this study. However, host feeding choice within a single species 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 (Savage et al. 2007; Kilpatrick et al. 2006; Apperson et al. 2004). Thus the exotic bird species used as bait, unknown to the local mosquitoes, would not be expected to be considered a preferential target. Also, if there was a preferential bird or mammal species nearby, any other used baits 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, they may play an important role in the local avian malaria transmission cycles, either as infection reservoirs (if they are infected) or as infection sinks (if they are malaria resistant). To our knowledge, no studies of haemosporidian infections have been conducted in Spotless Starling yet; however, its close relative Common Starling (*Sturnus vulgaris*) seems to be quite resistant against *Plasmodium* infections (Garamszegi 2011; Peirce 1981b). Only two lineages (GRW4 and TUMIG05), both without local transmission at the study sites (Ventim et al. in press-a), are reported for this species in its introduced range (Beadell et al. 2006; Martinsen et al. 2007); a European study failed to experimentally infect Common Starlings with SGS1 (Palinauskas et al. 2008). If the Spotted Starling is also a poor host for haemoparasites, but is a preferential target for their vectors, then it should contribute to break the parasite's dispersal cycle and thus lower the malaria transmission rate to other local bird species. This situation, in which vectors are monopolized by abundant yet incompetent vertebrate hosts that act as dead ends for effective transmission, is called zooprophylaxis (Dobson et al. 2006). This could also result in a low infection rate in Tornada's vectors, which was indeed observed.

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 the bodies of unengorged *Cx. pipiens* and *Cx. theileri* (respectively), suggesting that these 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 a bird species that may be a dead end in the transmission chain of avian malaria. To our knowledge, this is the first report of detection of avian *Plasmodium* DNA from European mosquitoes.

Acknowledgements

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GENERAL DISCUSSION

A Chiffchaff rests after surviving a blood donation.
Photo by: Rita Ventim (2007).

General discussion

This study provided valuable information on the transmission of avian haemosporidians in south-western European reed beds, focusing on the interactions between haemosporidians, their vectors and their avian hosts. An overview of the infection prevalence and intensity in several bird species is given in chapters 1 and 4, together with a study of which factors influence the prevalence and intensity of haemosporidian infections in each host species. With this work it was possible to understand better the haemoparasite´s specificity, the structure of the host-parasite interactions (chapter 2) and the geographical configuration of parasite assemblages (chapter 3). The local vectors and their relations both with parasites and with birds were also investigated (chapter 5).

The overall infection prevalence varied among the studied bird species, from 56% for Cetti´s Warbler *Cettia cetti* to zero detected infections in the Common Chiffchaff *Phylloscopus collybita*. Prevalence did not seem to be phylogenetically determined (at the genus level) and is probably more influenced by ecological, immune and physiological constraints (although phylogeny can play a role in defining the latter). Infections in the different host species were affected by distinct variables, according to the host's biology. Age was the most important factor for the Reed Warbler *Acrocephalus scirpaceus*, reflecting its migratory character: juveniles had a lower prevalence because they had only been exposed to the local parasites, whereas adult individuals had already migrated and accumulated parasite infections on both their wintering and reproductive sites (Hellgren et al. 2007b), causing a higher overall prevalence. For the resident Cetti's Warbler, prevalence varied significantly with season, increasing strongly from spring to autumn. Perhaps the lower food availability in the reed beds during autumn and winter made these birds weaker and more prone to infection, so the infections contracted during late summer were maintained during autumn and winter. For the House Sparrow *Passer domesticus*, a generalist species that can rely on many food sources, this tendency was more subtle.

The parasitemia of *Haemoproteus* spp. infections was significantly higher than that of *Plasmodium* spp., confirming the findings of Atkinson and van Riper III (1991). Nevertheless, all the infections had low level parasitemias, consistent with the chronic phase of infection (Zehtindjiev et al. 2008; Valkiūnas 2005). This only confirms that mist nets seldom catch heavily infected birds, which should be less mobile (Valkiūnas 2005). Therefore, the samples collected by this method cannot give us the complete picture of the natural levels of infection prevalence, infection intensity and fitness effects on the host.

Nine lineages of *Haemoproteus* and 15 lineages of *Plasmodium* were found, of which only ten *Plasmodium* were proven to have local transmission (because they occurred in resident species or juveniles from migrant species, all of which should have spent most of their lives in

the studied reed beds). The absence of biting midges (*Culicoides* sp.), *Haemoproteus* main known vectors, from the studied reed beds (R. Ventim, unpublished data) confirms that this parasite genus should not be transmitted locally. Therefore, all the detected *Haemoproteus* infections must have been contracted elsewhere. Contrarily, many *Plasmodium* lineages have both African and European transmission, as is the case for SGS1 (Hellgren et al. 2007b); so some of the *Plasmodium* lineages that were present in this community are expected to be transmitted locally as well as in Africa.

Each parasite lineage showed specific host preferences, instead of a random distribution in the different host species. Most lineages in this study were only detected in one of the analyzed bird species, indicating that these are relatively specialized parasites. The host-parasite interaction matrix was not nested, meaning specialists did not always share hosts with generalist parasites (Bascompte and Jordano 2007; Bascompte et al. 2003). In this way, specialist parasites can be free from the competitive pressure of generalist parasites by infecting bird species unexplored by the generalists.

In this bird community, *Plasmodium* SGS1 was the most prevalent lineage overall and also the most host-generalist. Although this parasite is a confirmed host-generalist, infecting birds of many different families (Hellgren et al. 2007b; Beadell et al. 2006), it seems not to be very successful infecting Reed Warblers and Great Reed Warblers *Acrocephalus arundinaceus* (Dimitrov et al. 2010; Zehtindjiev et al. 2008; Waldenström et al. 2002). The more host-generalist parasites in this study were those that reached a higher overall prevalence, and also reached high prevalence over a greater number of bird species, as was also found by Ricklefs et al. (2005) and Hellgren et al. (2009). Having a broad host range amplifies the prevalence in each host species (Hellgren et al. 2009) because the more prevalent lineages are transmitted to vectors more often and, if they are generalist parasites, they will be more likely to end up infecting a suitable host. In this way, the encounter rate between these parasites and all species in the bird community increases, leading to higher prevalence in all of the hosts (Hellgren et al. 2009).

However, caution is needed before classifying a parasite as a specialist or a generalist, since its apparent specialization can depend on the type of host species that are sampled. For example, parasites with narrow habitat preferences or geographical range may appear to be very host-specialist if sampling is made in the margins of that habitat or range, whereas they can appear to be generalists in samples taken from within that habitat or range. This was the case of *Haemoproteus* MW1, which seems to prefer marsh warblers as hosts, so it appeared to be more generalist in the studied reed bed communities than in studies involving hosts from other habitats (Krizanauskiene et al. 2006; Waldenström et al. 2002).

Site did not influence parasite prevalence for any host species at this small geographical scale, although effects on prevalence have been previously found at a wider geographical scale

(Bensch and Akesson 2003 in Sweden; Bennett et al. 1995 in Scandinavia; Merilä et al. 1995 throughout Europe). For two migratory species, the Reed Warbler and the Great Reed Warbler, parasite assemblages showed little geographical structure throughout Europe when the present data were compared with data from previous studies in other European sites. In these and other bird species with high migratory connectivity (Procházka et al. 2009; Yohannes et al. 2008), parasite assemblages could be expected to be structured by the host's migratory movements, perhaps to the point that they might be used as geographical markers (Webster et al. 2002). However, the present study found that the parasite lineages are homogenized by other processes. Generalist lineages (such as *Plasmodium* SGS1 and *P.* GRW04) may use several different host species to expand and maintain their geographical range independently of some host's migratory movements (Jenkins and Owens 2011); whereas specialist lineages (such as *Haemoproteus* GRW05) are much more dependent on the movements of their single or few host species and therefore confer some geographic structure to haemoparasite assemblages. Therefore, parasite assemblages as a whole show little geographic structure and appear to be of little use as indicators of migratory connectivity.

In the studied marshes, the colonizing exotic passerines (the Red Avadavat *Amandava amandava*, the Waxbill *Estrilda astrild*, the Black-headed Weaver *Ploceus melanocephalus* and the Yellow-crowned Bishop *Euplectes afer*) harboured less infecting lineages and at a lower prevalence than the native species. However, when phylogeny was controlled for, there were no significant differences between exotics and natives. Therefore, we found no evidence that the enemy release hypothesis (Torchin et al. 2003; 2001) applied to the haemoparasites of these exotic bird species. The parasite lineages of these birds' original home range (Durrant et al. 2007; Ishtiaq et al. 2007; Beadell et al. 2006) seem to have been lost before or after the birds' arrival to the study sites (MacLeod et al. 2010); but after that, instead of remaining free from haemoparasites, these exotic birds were colonized by local parasite lineages. In this way, release from haemoparasites did not seem to have a role in the colonization success of these exotic bird species. As all the studied exotic species were related and had a similar colonization process, it is not possible to know if parasitism may be important in deciding the outcome of colonization for other introduced birds, under different conditions.

Two local *Plasmodium* lineages infected the exotic bird species: one of them (SGS1) was the most prevalent lineage in the native species, so it could be expected to be transmitted to the exotics by chance; being such a host-generalist, it was expected to succeed in infecting these new hosts. The other lineage (PADOM01) was rarer in the sampled community, but was present in the native House Sparrow and seems to be specialized in Passeroid hosts. Therefore, its colonization of the exotic species seems to be favoured by phylogenetic factors.

In three of the study sites, the most abundant mosquito species were *Culex pipiens*, *Cx. theileri* and *Ochlerotatus caspius*, which were previously described as the most abundant

mosquitoes in Portugal (Osório et al. 2010; Almeida et al. 2008). At the Tornada site, *Coquillettidia richiardii* was very abundant, while *Cx. theileri* and *Oc. caspius* were absent. Because this site 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. Unengorged *Cx. pipiens* and *Cx. theileri* were infected (respectively) by *Plasmodium* SGS1 and *P.* SYAT05, two locally transmitted lineages. This suggests that those mosquitoes may be competent vectors of these lineages. Indeed, both mosquito species have already been described as vectors of avian *Plasmodium* elsewhere (Ejiri et al. 2011; Kim and Tsuda 2010; Kim et al. 2009; Valkiūnas 2005). These mosquito species were more abundant than expected in the same site that had a higher prevalence of SGS1, hinting that alterations in influence of vector abundance may influence variations of parasite prevalence.

When mosquito feeding preferences were evaluated using CO_2 and animal-baited traps, *Cq. richiardii* appeared to be an opportunistic feeder, while *Cx. pipiens* appeared to be mammophilic, contrarily to previous studies (Savage et al. 2007; Apperson et al. 2004). Perhaps this was because the used avian bait was not its preferential target, as mosquitoes that feed upon avian or mammal hosts target a limited number of species most of the time (Savage et al. 2007; Kilpatrick et al. 2006; Apperson et al. 2004). At Tornada, the identification of blood meals made of Spotless Starling *Sturnus unicolor* in engorged mosquitoes of several species suggests that this bird species is a preferential target for ornitophilic mosquitoes. Therefore, the Spotless Starling may play an important role as a local reservoir of avian malaria.

This work provided a complete overview of the host-parasite-vector system at the studied reed bed communities. This was the first study of haemosporidian transmission in south-western Europe including a community of 13 passerine species (counting in resident, migratory and exotic species). Extensive sampling of this kind adds to the accumulated knowledge of avian malaria infections, helping to appreciate presence and prevalence differences between distinct bird species, geographic regions or environmental conditions. It was also the first study of mosquitoes as possible avian malaria vectors in this region, as well as the first time in Europe that *Plasmodium* DNA was amplified from mosquitoes.

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