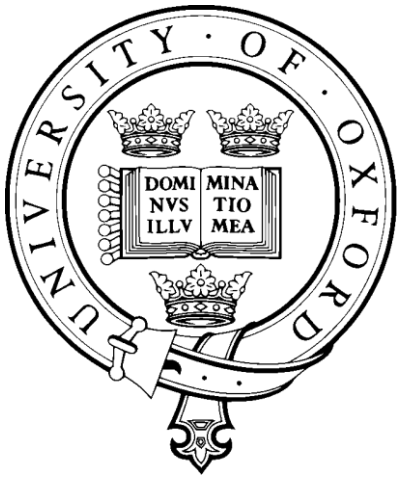
Avian malaria associations with British mosquitoes



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Abstract

Avian malaria associations with British mosquitoes
DPhil thesis by Ricardo Alves, St. Catherine's College, submitted Trinity Term 2012

Avian malaria (*Plasmodium* spp.) is a popular model system to study the ecology and evolution of parasite-host-vector interactions in the wild. These studies have historically focused mostly on the avian hosts and the malaria parasites. Knowledge regarding the role of vectors is essential to our understanding of these wild systems, but has only very recently started to accumulate. This thesis aimed to contribute to this field by assessing mosquito-malaria-host associations for British mosquitoes and the role of mosquito ecology in shaping these parasite systems in a British woodland study site, using molecular, field ecology and statistical modelling methodologies. From the 12 mosquito species or species groups found, I showed that the *Cx.pipiens*/*torrentium* mosquito group is likely to have a major role in avian malaria transmission in Great Britain, while *Cs. annulata* may be transmitting *P. circumflexum*. I also demonstrated a positive spatial association between mosquito density per host and avian malaria prevalence, in accordance with theoretical expectations for malaria transmission. Findings here provide evidence that avian malaria transmission in British woodlands is limited mainly to June-August, being preceded by relapse of previous infections or, alternatively, by maintenance of chronic blood parasitaemia through the colder months; this agrees with theoretical expectations and findings elsewhere for temperate climates. This thesis also described local-scale spatial heterogeneity and seasonal variation in adult mosquito abundance within a British woodland where avian malaria is endemic, with differing patterns found between species or species groups. Spatially, variation in adult mosquito abundance was associated with microclimatic and landscape variables such as distances to mosquito breeding sites, microclimate and canopy height; seasonally, variation in mosquito abundance was associated with temperature and rainfall, alongside calendar date. The heterogeneity in mosquito parameters and associations with environmental variables found at a site where avian malaria is endemic highlights the need to anticipate such complexity when trying to understand *Plasmodium* transmission. By doing so, we further extend the potential of these parasite systems to improve our knowledge regarding the ecology and evolution of parasite-host-vector associations.

Acknowledgements

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Appendix I to IV: papers published during DPhil to which R. Alves contributed key data

Introduction

Malaria parasites (Apicomplexa: Haemosporida) are vector-borne and can infect reptiles, birds and mammals, including humans (Garnham, 1966). Annually, human malaria results in an estimated 250 million clinical cases and at least one million deaths (WHO, 2008). These parasites also have veterinary importance (Valkiūnas, 2005). Their importance for human and animal health has led to a large body of work on malaria parasite-host-vector interactions. These have mostly focused on human malaria, but avian malaria played a very important historical role as a model system. Ronald Ross, who was awarded a Nobel Prize for his groundbreaking work, was the first to ascertain the malarial parasite's life cycle, doing so by working with the avian parasite *Plasmodium relictum*. Research on chemical therapy (Wasielewski, 1904) and cultivation *in vitro* (Hawking, 1944) was first successfully achieved using avian malaria models. Today, avian malaria parasites are still a relevant and low cost model to inform human malaria, in particular for immunological, genetic, and biochemical studies (Valkiūnas, 2005).

Avian malaria has also gained popularity as a model system to study parasite-host-vector interactions in the wild, particularly in relation to their importance for understanding sexual selection (e.g. Hamilton & Zuk, 1982), life-history trade-offs (e.g. Richner *et al.*, 1995), sex allocation (e.g. Merino *et al.,* 2004), parasite community ecology (e.g. Ricklefs *et al.*, 2005), speciation (e.g. Pérez-Tris *et al.*, 2007), landscape and host effects on parasite prevalence (e.g. Wood *et al.*, 2007) and parasite-vector specificity (e.g. Njabo *et al.*, 2009). The majority of this work has focused on vertebrate host-malaria interactions, with the vectors of avian malaria remaining largely unstudied in wild conditions (Valkiūnas, 2005); only very recently has this scenario started to change. Given the essential role of vectors in

the malarial life cycle, such knowledge is needed to understand avian malaria in the wild. This thesis focuses on mosquito ecology and their associations with avian malaria parasites and hosts in wild British populations. In this chapter, relevant aspects of avian malaria parasites and their vectors will be introduced, followed by the main study site and host populations considered herein. The research questions addressed by this thesis end this chapter.

Systematics, distribution, life cycle and pathology of avian malaria

Systematics

Avian malaria parasites (*Plasmodium* spp., *sensu* Valkiūnas *et al*., 2005) are included in the order Haemosporida, along with three other genera of apicomplexan parasites known to infect birds: *Haemoproteus*, *Leucocytozoon* and *Fallisia*. Recent developments in molecular detection techniques have revealed approximately 300 unique avian *Plasmodium* lineages, based on a 478bp fragment of the cytochrome *b* gene (Waldenström *et al.,* 2004), while a single lineage may occur in up to 45 different avian host species (Bensch *et al.*, 2009).

Distribution

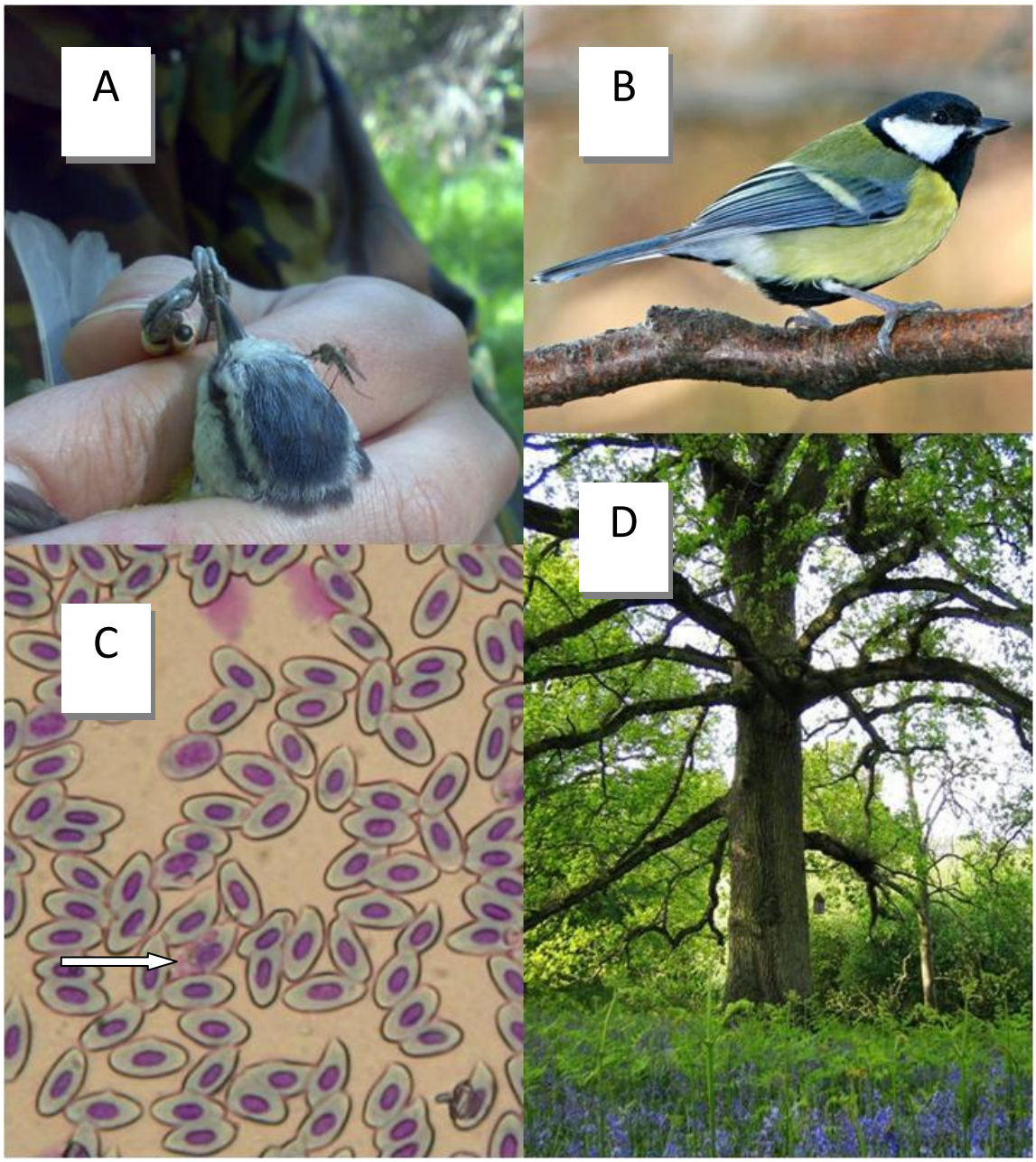
Avian *Plasmodium* is present in every zoogeographical region, except the Antarctic where there are no records (Valkiūnas, 2005). Several *Plasmodium* species are known to occur in up to three or four different geographical regions (e.g. *P. relictum* and *P. circumflexum*) (Valkiūnas, 2005). Around 45% of bird species in the world have been screened for

haemosporidian infection, with approximately 30% of screened species being proven hosts of *Plasmodium* (Valkiūnas, 2005).

Life cycle

The life cycle of all haemosporidians requires a vertebrate host and an invertebrate host which acts as vector (blood-sucking dipterans, Insecta: Diptera). The general form of this life cycle is quite conserved, although differences may occur even between members of the same genus of haemosporidians. When a vector takes a blood meal on an infected vertebrate host, it may pick up macro and micro gametocytes. Inside the vector gut, these release macro and micro gametes, which fuse to form a diploid zygote, which develops into a motile ookinete. These enter the basal lamina of the gut, forming an oocyst, where formation of sporozoites (haploid) takes place. Sporozoites then migrate to the salivary glands, from where they can be injected into a vertebrate host during the next blood meal. Depending on the haemosporidian species, sporozoites invade cells of different tissues: lungs, kidney, spleen, skeletal muscle and reticulo-endothelial cells. Asexual replication then follows to produce merozoites, which invade circulating blood cells. In *Plasmodium* only, merozoites can develop into meronts inside erythrocytes, leading to further merozoite production. Merozoites can differentiate into macro or micro gametocytes (Figure 1.1) that erupt from the blood cells and circulate in the blood stream, becoming once again available to be picked up in the vector blood meal (Valkiūnas, 2005). The period required for all *Plasmodium* stages to occur in mosquito hosts is species and temperature dependent, and may last only six days; in the avian host, this period is species dependent and typically lasts

Figure 1.1 - (A) Blue tit with mosquito probing in search of a blood meal in Wytham Woods. (B) Great tit. (C) *Plasmodium* sp. gametocytes inside avian (*M. striaticollis*) erythrocytes (1250 x). (D) View within Wytham Woods, a temperate mixed deciduous woodland showing nest box suspended from oak.



(Photo credit for B: Joe Tobias; for C: Ben Daly; for D: Matt Wood)

a minimum of seven to ten days (Valkiūnas, 2005). The life cycle of avian *Plasmodium* genera share most similarities with human malaria parasites, including mosquitoes (Diptera: Culicidae) as vectors and the erythrocytic stage of merozoite replication, with these two malaria parasite groups regarded as sister taxa (Outlaw, 2011).

Pathology and immunity

Plasmodium sp. pathology is caused by exoerythrocytic and blood stages of these parasites, which can cause inflammatory reactions, capillary blockage of vital organs, destruction of blood cells and anaemia (Valkiūnas, 2005 and references therein). Malaria parasites can cause decreases in productivity and even high mortality in domestic and captive birds, with symptoms including weakness, worsening of motor coordination and paralysis (Garnham, 1980; Huchzermeyer, 1993; Valkiūnas, 2005). Experimental and comparative studies in wild populations revealed that chronic infections with *Plasmodium* can negatively affect bird fitness (Knowles *et al.*, 2010a; Ashgar *et al*., 2011; Lachish *et al*., 2011a; Reed *et al*., 2012). Cases of severe pathology in wild birds caused by these parasites have been recorded (Manwell, 1951; Garnham, 1966). Following studies in captive birds, the introduction of *Plasmodium relictum* in Hawaii was implicated in the widespread decline and the possible extinction of many species (van Riper III *et al.*, 1986; Atkinson *et al.*, 2000). Taken together, these studies suggest significant effects of avian *Plasmodium* infection in wild birds, with consequences for their demography, ecology and evolution.

Avian host immunity for *Plasmodium* remains understudied (Valkiūnas, 2005). Antibodies against *Plasmodium* were found in avian hosts, including in birds where malaria parasites are not detected (Jarvi *et al*., 2002; Sturrock & Tompkins, 2007). Acquired immunity

against *Plasmodium relictum* was found experimentally in avian hosts subject to secondary infection with the same malaria parasite morphospecies (Atkinson *et al*., 2001). In the wild, mixed infections are common (e.g. Ishtiaq *et al*., 2006; Bensch *et al*., 2007) and secondary infection with a different *Plasmodium* morphospecies can occur after infection loss (Lachish *et al*., 2011b), suggesting acquired immunity (if occurring) does not protect against different *Plasmodium* parasites. Theoretically, chronic *Plasmodium* infections in avian hosts showing low parasitaemia and absence of discernible pathology are thought to be maintained by acquired immunity, which may reduce parasitaemia but not completely clear infections (Manwell, 1934; Atkinson & van Riper, 1991; Jarvi *et al*., 2002; Valkiūnas, 2005).

Avian malaria vectors

The work of Ronald Ross revealed the need of an invertebrate vector for the completion of haemosporidian life cycle and transmission between vertebrate hosts. Avian haemosporidian vectors are blood-sucking insects from the Order Diptera. Most of the different haemosporidian genera are associated with different Dipteran families: Culicidae (mosquitoes), Cerapotogonidae (midges), Simuliidae (black flies) and Hippoboscidae (louse flies) (Valkiūnas, 2005). Avian *Plasmodium* is transmitted by mosquitoes only (Valkiūnas, 2005). If a vector acquires *Plasmodium* gametocytes, they may transmit them to a new vertebrate host if the extrinsic incubation period (EIP) of the malaria parasite inside the vector has occurred between any given two blood meals. The EIP varies between mosquito and malaria parasite strains and may last only 6 days under optimal temperature,

with sub-optimal temperatures lengthening or arresting malaria parasite development (Valkiūnas, 2005 and references therein; LaPointe *et al*., 2010).

Mosquito biology can vary between species. Eggs can be laid in water, soil or organic matter, but require an aquatic environment with low or no movement to function as a breeding site to complete the stages of immature development, i.e. egg, four larval stages and pupae (Service, 1980). Adult (imago) mosquitoes typically mate within one day after emergence and only the female feeds on vertebrate blood (in order to obtain nutrients for egg development) and can therefore act as a malaria vector (Gillet, 1972). Females do not lay eggs continuously, typically following a gonotrophic cycle consisting of response to vertebrate host stimulus, blood-feeding, egg development and oviposition. The dynamics of mosquito life cycle and survival rate are critically dependant on climate variables (Rogers & Randolph, 2006). Temperature typically decreases mosquito longevity and the duration of the gonotrophic cycle (Service, 1980), low and high humidity influences mosquito survival (Clements, 1992) and rainfall affects breeding site availability and suitability for immature development (Juliano, 2009). In temperate climates, detrimental climatic conditions cause many mosquitoes to enter diapause (i.e. suppressed development, usually preceding the onset of detrimental conditions), which may not terminate with the end of adversity (Koštál, 2006). In temperate climates, females can live more than six weeks and mosquito populations may have a limited number of generations each year (species are univoltine, with a single generation per year, bivoltine or multivoltine) (Service, 1980).

Avian malaria-mosquito associations

Plasmodium vector research has largely focused on human malaria, to the extent of making mosquitoes one of the most intensively studied groups of the Animal Kingdom, leading to a considerable body of knowledge regarding their distribution (e.g. Becker *et al*., 2010), life cycle (e.g. Clements, 1992), and epidemiological interactions with human hosts, malarial parasites and environmental variables (e.g. MacDonald, 1957; Takken *et al*., 1990). Such insights for human malaria have greatly aided in the understanding and control of this disease, with vector control regarded as the generally most effective method to prevent transmission (WHO, 2006). Knowledge available regarding avian *Plasmodium* vectors is much less extensive, with vector species not determined for many parasite species and most available knowledge being obtained in laboratory infection studies (Valkiūnas, 2005). For avian *Plasmodium*, this scenario has started to improve only recently, with an upsurge of novel work being done on the identity of mosquito species associated with these malaria parasites in the wild (Table 1.1) and the avian host preferences of these species determined using molecular methods (Huijben *et al.,* 2007; Ejiri *et al.,* 2008; Gager *et al.,* 2008; Ishtiaq *et al.,* 2008; Ejiri *et al.,* 2009; Kim *et al.,* 2009a; Kim *et al.,* 2009b; Massey *et al.,* 2007; Njabo *et al.,* 2009; Bueno *et al.,* 2010; Hughes *et al.,* 2010; Kim & Tsuda, 2010; Kimura *et al.,* 2010; Carlson *et al.,* 2011; Ejiri *et al.,* 2011a; Ejiri *et al*., 2011b; Njabo *et al.,* 2011; Glaizot *et al*., 2012; Inci *et al*., 2012; Ventim *et al*., 2012). The methodologies used typed different loci in the malaria parasite mitochondrial DNA, obtaining lineages that can correspond to the same morphospecies, i.e. a group of individuals morphologically similar and clearly separated from other groups by morphology (see http://mbio-serv4.mbioekol.lu.se/avianmalaria/index.html) for information on avian malaria parasite

morphospecies ascribed to loci). These studies have generally shown that only a small proportion of mosquito species are associated with avian *Plasmodium* and in low prevalence (usually 1-5%, but may approach 10-30%) (Table 1.1). This is similar to the situation with human *Plasmodium* (Dye, 1992). In accordance with experimental studies, *Plasmodium* parasites have been found in more than one mosquito genus in the wild, unlike human malaria which is transmitted by *Anopheles* (*An.*) only. The genera associated with avian malaria are *Aedeomya* (*Ad*.), *Aedes* (*Ae*.), *Culex* (*Cx*.), *Coquillettidia* (*Cq*.), *Culiseta* (*Cs*.), *Lutzomia* (*Lt.*) and *Mansonia* (*Ma*.). Overall, this indicates that avian malaria transmission is being undertaken by a small proportion of the total mosquito fauna, but using a wider vector breath than observed for human *Plasmodium*. These associations remain largely unstudied in many habitats and geographic regions, including the European landmass, with the exception of work done in a zoological garden in the Netherlands (Huijben *et al.*, 2007) and in several Iberian (Ventim *et al*., 2012) and Alpine (Glaizot *et al*., 2012) habitats. Therefore, one aim of this thesis was to uncover mosquito-avian host-malaria parasite associations in wild caught British mosquito specimens.

Table 1.1 - Mosquito-avian *Plasmodium* associations uncovered in the wild. Mosquito genus: *Aedeomya* (*Ad*.), *Aedes* (*Ae*.), *Culex* (*Cx*.), *Coquillettidia* (*Cq*.), *Culiseta* (*Cs.*), *Lutzomia* (*Lt.*) and *Mansonia* (*Ma*.). "N" and "N pools"= number of specimens and pools, respectively, of a species or species group screened. "MIR" = minimum infection rate (number of pools positive for *Plasmodium* / number of mosquitoes screened) × 100 (%). "MLE" = maximum likelihood estimation, per 100(%) mosquitoes, based on pool size, of the proportion of infected individuals in pooled samples; lower, upper, given 95% confidence limits in square brackets. "*Plasmodium* lineages found" = parasite lineages found in a mosquito species or species group, with known corresponding morphospecies in brackets.

Location and source paper	Mosquito species or species group	N	N pools	*Plasmodium* positive	Body part type screened	*Plasmodium* lineages found
Rotterdam, The Netherlands Huijben *et al.,* 2007	*Cx. pipiens*	348	NA	1.7%	Whole specimen	NA
Gisborne, New Zealand Massey *et al.,* 2007	*Cx. pervigilans*	1	1	1 abdomen	Blood-fed abdomen	NA
Minami Daito Island, Japan Ejiri *et al*., 2008	*Cx .quinquefasciatus*	1066	294	1.1(MIR, %)	Whole specimen	AB3008044-5 AB308048 AB308049 (*P. gallinaceum*) AB30850-1
	Ae. albopictus	81	46	1.2(MIR, %)		AB308047
	Lt. fuscanus	5	5	20.0(MIR, %)		AB308046
	Mansonia spp.	36	20	2.8(MIR, %)		AB308052
Central Panama Gager *et al.,* 2008	*Ad. squamipennis*	780	39	12 pools	Whole specimen	PAN PAN4-6 PAN8-9
	Cx. ocossa	1520	76	9 pools		PAN2-3 PAN7
Vanuatu and New Caledonia Ishtiaq *et al.,* 2008	*Ae. hebrideus*	14	3	Thorax and abdomen: 12.83(MLE, %) [0.56, 52.46]	Thorax and abdomen	LIN2p
	Ae. notoscriptus	1	NA	1 abdomen		LIN3p
	Cx. sitiens	130	Thorax 10 Abdomen: 7	Thorax: 1.55(MLE, %) [0.29, 4.54] Abdomen: 2.90(MLE, %) [0.560, 8.0]		LIN1p
	Cx. annulirostris	28	Thorax: 4 Abdomen: 2	Thorax: 20.6(MLE, %) [4.58, 50.6] 2 abdomen pools		LIN1p LIN4p (*P. juxtanucleare)*
Kanagawa, Japan Ejiri *et al.,* 2009	*Cx. pipiens* group	763	63	0.52(MIR, %)	Whole specimen	AB474376 AB474377 (*P. relictum*)
	Lt. vorax	78	12	5.13(MIR, %)		AB474378-9 AB474381 AB474382 (*P.relictum*)

Tokyo, Japan Kim *et al.,* 2009a	*Cx. pipiens pallens*	368	46	2.99(MIR, %)	Whole specimen + blood-fed abdomen	Yacho-1 Rinshi-1 Rinshi-3 Rinshi-8 (*P. relictum*)
	Cx. pipiens molestus	74	12	1.35(MIR, %)		Rinshi-3
	Lt. vorax	1	1	100(MIR, %)		Rinshi-3
Tokyo, Japan Kim *et al*., 2009b	*Cx. sasai*	21	NA	3 specimens	Blood-fed abdomen	Rinshi 1-3
South Cameroon Njabo *et al.* 2009	*Cq. aurites*	1082	220	8.40 (MLE, %)	Thorax	PlasCoq1-2 PlasCoq4-6 PV11 PV12
	Cq. pseudoconopas	183	31	3.55(MLE, %)		PV12
	Cq. metallica	20	5	11.22(MLE, %)		PlasCoq3 PV12
São Paulo, Brazil Bueno *et al.,* 2010	*Culex sp.*	10	NA	1 positive	Whole specimen	HM242416 HM242417
Madison, USA Hughes *et al.,* 2010	*Cx. pipiens/restuans*	116	NA	11.5 %	Whole specimen (gravid)	NA
Tokyo, Japan Kim & Tsuda, 2010	*Cx. pipiens pallens*	881	95	32 pools	Abdomen (unfed)	CXPIP09 SGS1 PADOM02
		371	NA	18.6%	Abdomen (blood-fed)	Rinshi-9 GRW11 Rinshi-5-6
		371	NA	3.0%	Thorax (blood-fed)	Rinshi-10 SYAT05 GALLUS01 (*P. gallinaceum*)
						In thorax : CXPIP09 SGS1 PADOM02 GALLUS01
Ithaca, USA Kimura *et al.,* 2010	*Cx. pipiens*	303	NA	14.2%	Whole specimen	SYAT05 LINN1, CXPIP01-06 TUMIG3 SEIAUR01
	Cx. restuans	180	NA	11.1%		SYAT05 LINN1 TUMMIG3 SEIAUR01 CXRES04 PADOM11 E1 CXRES01-06
	Ae. canadensis	161	NA	0.6%		SEIAUR01
Socorro Island, México Carlson *et al.,* 2011	*Cx. quinquefasciatus*	11	1	Abdomen pool positive	Thorax and abdomen	SocP11
	Ae. taeniorhynchus	1220	61	Thorax : 0.08(MLE, %) Abdomen: 4.8(MLE, %)		SocP1-10
Hokkaido, Japan Ejiri *et al.,* 2011a	*Cx. pipiens* group *Ae. esoensis*	193043	NANA	1 pool positive3 pools positive	AbdomenThorax or abdomen (blood-fed	Kushiro-4Kushiro-1 Kushiro-2 Kushiro-3 (*P. gallinaceum*)

Tokyo, Japan Ejiri *et al*., 2011b	*Cx. pipiens* group	NA	41	4.3(MIR, %)	Whole specimen	AB542062 (*P. relictum*) AB542064
	Cx. pipiens pallens	131	NA	16%	Blood-fed specimens	AB542061-8
South Cameroon Njabo *et al.,* 2011	*Ae. mcintoshi*	277	25	1.52 (MLE, %) [0.51, 3.64]	Thorax	PV11
	Cq. aurites	1118	230	10.52(MLE, %) [8.65, 12.67]		*Coquillettidia* spp: PlasCoq1-6 PLasCoq13-14 PlasCoq16
	Cq. metallica	21	6	10.69(MLE, %) [2.00,33.29]		PV11-2
	Cq. pseudoconopas	184	32	7.13(MLE, %) [3.81, 12.27]		
	Cx. annulioris var. major	66	8	3.04 (MLE, %) [0.58, 9.69]		*Culex* sp: PlasCoq3-4 PlasCoq6-12 PlasCoq16
	Cx. neavei	138	16	7.56 (MLE, %) [3.63, 14.45]		
	Cx. perfidiosus	138	16	6.26 (MLE, %) [2.85, 12.35]		
	Cx. poicilipes	25	3	10.02(MLE, %) [2.02, 40.02]		
	Cx. guiarti	335	37	0.29 (MLE, %) [0.02, 1.43]		
	Ma. uniformis	515	32	0.39 (MLE, %) [0.07, 1.28]		PlasCoq15
Lausanne, Switzerland Glaizot *et al*., 2012	*Cx.pipiens*	394	NA	6.6%	Whole specimen	SGS1 (*P. relictum*) GRW11 (*P. relictum*) P5 (*P. relictum*) TURDUS1 (*P. circumflexum*) SW2 (*P. polare*) SYAT05 (*P. vaughani*)
Central and South Portugal Ventim *et al*., 2012	*Cx. pipiens*	565	NA	0.35(MIR, %)	Whole specimen	SGS1 (*P. relictum*)
	Cx. theileri	372	NA	0.27(MIR, %)		SYAT05
	Cx. peregxiguus	11	NA	9.09(MIR, %)		NA
Kayseri Province, Turkey Inci *et al*., 2012	*Ae. vexans*	NA	Thorax and abdomen: 312	Thorax: 4.72 (MIR, %) Abdomen: 5.98(MIR, %)	Thorax and abdomen	Kayseri1
	Cx pipiens	NA	Thorax and abdomen: 241	Thorax: 16.22(MIR, %) Abdomen: 18.15 (MIR, %)		KYS3-8 KYS11 Kayseri2
	Cx. theileri	NA	Thorax and abdomen: 18	Thorax: 5.18(MIR, %) Abdomen: 10.36(MIR, %)		KYS10
	Cs. annulata	NA	Thorax and abdomen: 15	Thorax and abdomen: 10.64(MIR, %)		KYS9

Species or species groups tested and found negative for *Plasmodium: Ae. aegypti, Ae. alternans, Ae. aurifer, Ae. bekkui, Ae. crinifer, Ae. daitensis, Ae. excrucians, Ae. flavopictus, Ae. galloisi, Ae. japonicus, Ae. nipponicus, Ae. punctor/hokkaidensis, Ae. scapularis, Ae. togoi, Ae. vigila, Ae. yamadai, Ae. domesticus, Ae. microstictus, An. albuminus, An. algeriensis, An. atroparvus, An. claviger, An. evansae, An.*

farauti, An. maculipennis, An. plumbeus, An. sinensis, An. coustani, An. funestus group*, An. gambiae* complex*, An. hancocki, An. nili, Ar. subalbatus, Cq. chrysonotum/albifera, Cq. nigricans, Cq. richiardii, Cq. xanthogaster, Cs. alaskaensis, Cs. fumipennis, Cs. inatomii, Cs. kanayamensis, Cs. melanura, Cs. morsitans, Cs. nipponica, Cs. subochrea, Cx. vansomereni, Cx. ameliae, Cx. bitaeniarhynchus, Cx. coronator* group*, Cx. erraticus, Cx. gaufini, Cx.habilitator/pseudojanthinosoma, Cx. hortensis, Cx. melanoconion* section*, Cx. modestus, Cx. nigripalpus, Cx. orientalis, Cx. restuans/declarator, Cx. rubithoracis, Cx. territans, Cx. torrentium, Cx. tritaeniorhynchus, Cx. vagans, Er. chrysogaster, Ho. psectropus, Lt. tigripes, Ma. dyari, Ma. indubitans, Ma. titillans, Oc. caspius, Oc. detritus, Or. anopheloides, Tp. bambusa, Ur. apicalis, Ur. iowi, Ur. novobscura, Ur. unguiculata* and *Ve. lineate.* A total of 23.8% of studies considered above only test a sub-group of species or species groups collected for *Plasmodium.*

Avian malaria vector ecology

Very few studies have looked into the role of mosquito ecology in shaping patterns of avian *Plasmodium* prevalence in birds. For other vector-borne parasites, vector distributions maps have been used to predict landscape-level disease prevalence (Ali *et al.*, 2003; Kulkarni *et al.*, 2010), and an effort is being undertaken to map the distribution of human malaria (*Plasmodium* spp.) vectors worldwide, regarded as a crucial tool for disease control (Hay *et al.*, 2010). Positive spatial associations between human malaria prevalence and vector abundance have been reported (Trape *et al.*, 1992; Ghebreyesus *et al.*, 1999; Clarke *et al.*, 2002; Galardo *et al.*, 2009), but also the absence of such a relationship (Thomson *et al.*, 1994; Diuk-Wasser *et al.*, 2005). The entomological inoculation rate (EIR) is a metric that estimates the number of infective mosquito bites per host per time unit (MacDonald, 1957). The EIR is usually used as a measure of transmission intensity and is positively associated with *Plasmodium* parasite prevalence in human malaria, although this relationship is complex (Beier *et al.*, 1999; Smith *et al.*, 2005). The EIR is not a function of mosquito abundance, but of mosquito feeding frequency, proportion of mosquitoes with malaria parasites in their salivary glands (sporozoite rate) and mosquito density per host (Hay *et al.*, 2000; Kelly-Hope & McKenzie, 2009). Pioneering work has been done previously looking into the relationship between mosquito distribution and/or abundance and avian *Plasmodium* prevalence. A widely cited model was proposed by van Riper III (1986),

which predicted intensity of avian *Plasmodium* transmission (and strong fitness costs to native birds) by overlaying over an altitudinal gradient the distributions of avian hosts and mosquito abundance in Hawaiian forests. Further work on this study system showed a higher proportion of malaria parasite infected mosquitoes, but not highest mosquito abundance, in the area with highest malaria parasite prevalence in birds. The authors speculated that this was due to coupling of a higher abundance of avian hosts capable of carrying chronic infections with year-round presence of mosquitoes in the area with the highest malaria parasite prevalence in both mosquitoes and birds (Woodworth *et al.*, 2005). Tompkins & Gleason (2006) reported a decrease of *Plasmodium* prevalence in avian hosts from north to south of the New Zealand landmass, matching the known vector distribution. Ventim *et al*. (2012) found no association between putative vector abundance and avian host *Plasmodium* prevalence in Portuguese reedbeds. Overall, these studies highlight the need to consider and understand vector ecology when looking into patterns of avian *Plasmodium* prevalence and avian host-malaria parasite co-evolutionary dynamics. Also, they show that like human malaria a simple positive relationship between vector abundance and avian *Plasmodium* prevalence in birds need not occur. Thus, another aim of this thesis was to look into a) predictors of spatiotemporal patterns of abundance for putative avian *Plasmodium* vectors in a British woodland where avian malaria is endemic (see below) and b) the relative importance of mosquito ecology, alongside environmental variables, in shaping spatiotemporal patterns of avian *Plasmodium* prevalence in birds, taking into consideration the concept of EIR.

Main study site and avian host-malaria parasite system

Wytham Woods

Wytham Woods is a mixed deciduous woodland (385ha) near Oxford, United Kingdom (51º46'N, 1º20'W). The site is situated on a hill within a loop of the Thames River which closely borders part of its northern limits, with the majority of the immediately surrounding areas being agricultural and pasture land. Nestboxes were first added in a selected area in 1947 aiming to study the great tit population, then throughout the rest of the wood between 1957 and 1965, and finally an addition of around 200 nest boxes specifically for blue tits brought the total number of boxes to approximately 1160 (Figure 1.2). Nestboxes were recently mapped with GIS techniques, with assessment of their altitude and distance to edge of wood (see Wilkin *et al*., 2007 for details). The majority (43-78%) of nestboxes are usually occupied by tits during the breeding season (typically April-early June), which allowed for a comprehensive gathering of data on their breeding ecology since 1947, by members of the Edward Grey Institute (EGI). The woodland supports a wide variety of bird and mammalian fauna, which are the source of blood meals for mosquitoes. Part of this vertebrate fauna has been the subject of studies regarding long-term monitoring of environmental changes and ecological processes (Savill *et al.*, 2010). Nectar and resting sites for mosquitoes are also widely available within and around the wood (Figure 1.1). Ponds, artificial water containers, temporary ground pools, water-filled tree holes and the close vicinity of the River Thames and its associated water bodies provide breeding site possibilities for mosquitoes, within and in the close proximity to the wood.

Investigations prior to the planning and execution of this thesis uncovered the occurrence of 7 species of mosquitoes assigned to the genera *Culex*, *Aedes* and *Culiseta* at this site collected by sweep netting, with overall mosquito abundance varying between different areas of the wood and being positively associated with overall avian malaria prevalence in blue tits (M.J. Wood, D.A. Cullen, R.M. Mallis, B.C. Sheldon, unpublished). This small scale investigation provided first insights into a possible role of mosquito ecology in shaping spatial avian malaria prevalence patterns, a question that will be addressed in more detail in this thesis.

Figure 1.2 - Aerial photograph of Wytham Woods, Oxford, United Kingdom. Location of all nestboxes shown by yellow dots (figure reproduced from Knowles, 2009).

The avian hosts

The blue tit (*Cyanistes caeruleus*) and the great tit (*Parus major*) are small birds from the order Passeriformes (Figure 1.1) with similar ecological requirements and breeding behavior (Perrins, 1979). They are common in the West Palearctic and abundant in European woodlands and gardens (Perrins, 1979). They readily breed in nestboxes, typically between April-June, and can do so at high densities (Barnes, 1975). Typically, the female lays one egg daily and starts incubation after completing a clutch (Wytham Woods mean clutch size: 8.9±3.4 eggs; minimum incubation period: 12 days; data for 2004-2008 blue tits, from Knowles, 2009). Both parents feed the chicks for approximately three weeks until fledging, which usually occurs between mid-May to mid-June. During the breeding season, they forage in the immediate vicinity of their nest (Stauss *et al.*, 2005). Within Wytham Woods, both species rarely produce more than a single brood a year, and show a relatively small median breeding dispersal distance (i.e. distance between successive breeding sites): 51m for blue tits (Knowles, 2009) and 143m being the highest median value recorded for great tits (Harvey *et al.*, 1979). Median natal dispersal distance is larger: 515m and 895m for male and female blue tits (Knowles, 2009), and 558m and 879m for male and female great tits (Greenwood *et al.*, 1979). Great tit recruits are known to have established home ranges broadly similar in location to their subsequent breeding territories by August (T.A. Wilkin, unpublished).

In Wytham Woods, between 250 and 450 pairs each of blue tits and great tits breed annually (Perrins 1979), with breeding pairs occupying nestboxes throughout the wood. From 2001 onwards, both the blue tit and great tit populations have been comprehensively monitored simultaneously during the breeding season, with approximately 80% of females

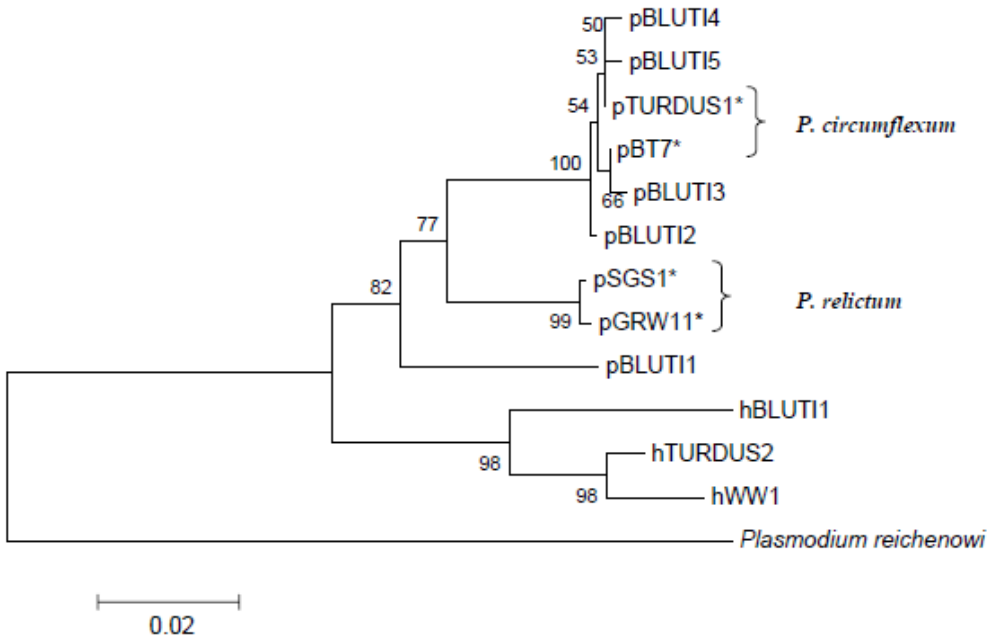
and 70% of males known to be feeding the chicks being captured. These are ringed, sexed and morphometrically assessed. Capture of chicks and sampling of breeding adults occurs between day 6 and day 14 of the nestling phase (usually from mid-May to early June).

Avian malaria in Wytham Woods

Adult breeding blue tits and great tits from Wytham Woods have been blood sampled and screened for avian malaria parasites in 2001, 2003-2010 and 2008-2009 respectively (Wood *et al.,* 2007; Cosgrove *et al.,* 2008; Knowles *et al.,* 2011; S. Lachish *et al.,* 2012). Thus, breeding avian hosts have been comprehensively blood sampled at a standardized point in their annual cycle, and the high synchrony in breeding attempts for both populations results in little variation in calendar date of sampling between years. All year round blood sampling in blue tits was done between 2003-2005 (Cosgrove *et al*., 2008). Screening of these blood samples has provided valuable insights into the diversity and spatiotemporal distribution of avian *Plasmodium* in Wytham Woods. For blue tits, detection by polymerase chain reaction (PCR) provided *Plasmodium* prevalence estimates of approximately 30% at Wytham Woods, during the breeding season (Wood *et al.,* 2007; Knowles *et al.,* 2011) or throughout the year (Cosgrove *et al.,* 2008). Amplification and sequencing of a 450 bp region of the cytochrome *b* gene (Waldenström *et al.,* 2004) has revealed four relatively common *Plasmodium* lineages in blue tits, with SGS1 being the most abundant (prevalence 10.2%), followed by TURDUS1 (9.7%), BT7 (4.6%) and GRW11 (1.3%) (Wood *et al*., 2007); other lineages constitute 2% of all infections found. The four most common lineages fall in two different clades (Figure 1.3) separated by approximately 7% sequence divergence at the cytochrome *b* locus, and correspond to two morphospecies, *P. relictum*

(lineages SGS1 and GRW11) and *P. circumflexum* (lineages TURDUS1 and BT7) (Palinauskas *et al.*, 2007). Use of quantitative PCR (on the same target region) able to detect both morphospecies increased *Plasmodium* prevalence estimates to approximately 40% for breeding blue tits, because of the higher sensitivity of the methodology (Knowles *et al.*, 2011). For great tits, the use of the same quantitative PCR methodology provided

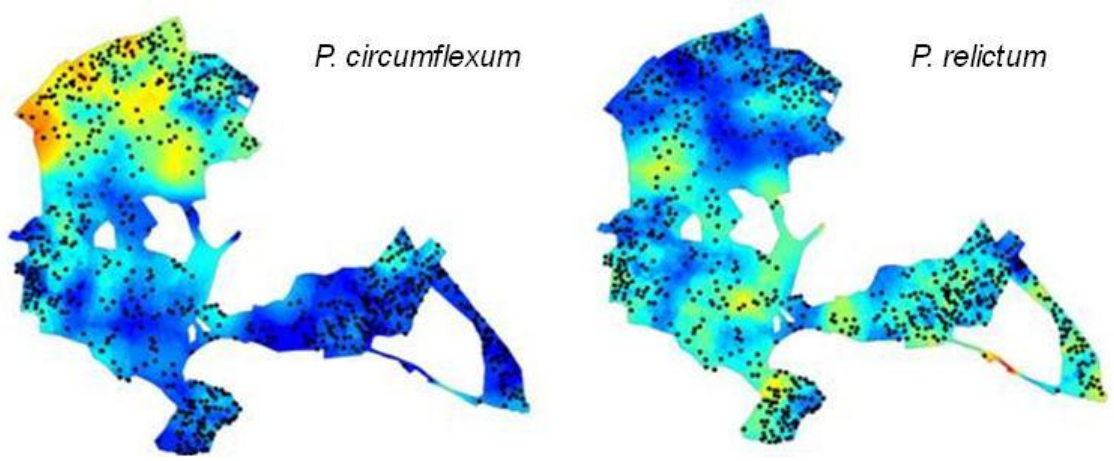
Figure 1.3 - Phylogenetic tree of avian haemosporidian lineages in blue tits in Wytham Woods. The neighbor-joining method was employed, with a Kimura 2-parameter distance matrix in MEGA 2.1 and using *Plasmodium reichenowi* (primate malaria) as the outgroup. Common lineages (*, >4% prevalence) fall in two different clades separated by approximately 7% sequence divergence at the cytochrome *b* locus, and correspond to two morphospecies, *P. relictum* and *P. circumflexum* (Palinauskas *et al.*, 2007) (figure reproduced from Knowles (2009), a previous adaptation from Wood *et al*., 2007).



Plasmodium prevalence estimates of approximately 50% (S Lachish *et al.*, 2012). *P. relictum* and *P. circumflexum* are placed in different subgenera, being distinguishable morphologically by gametocyte shape and pattern of cytoplasmic pigment distribution; they

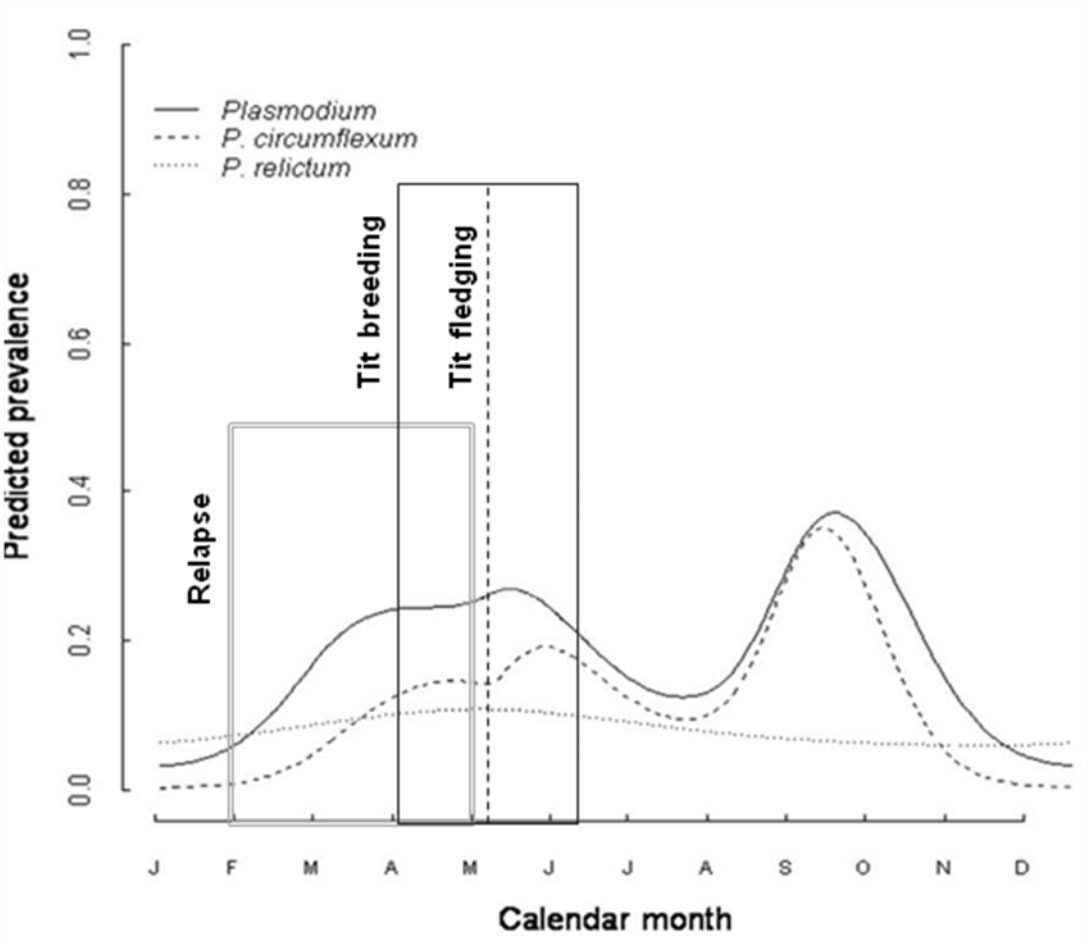
present differences in vector range and development in avian hosts (e.g. host range and synchronization of erythrocytic merogony) (Valkiūnas, 2005). In Wytham Woods, these malaria parasites are known to influence avian host survival and recapture rates (Lachish *et al*., 2011a), reproductive trade-offs between the number and quality of offspring (Knowles *et al*., 2010b) and behaviour traits such as exploration, problem-solving and startle response (Dunn *et al*., 2011). *Plasmodium* prevalence is highly spatially heterogeneous in Wytham Woods. For *P. circumflexum*, infection risk in breeding blue tits (2005-2010) and great tits (2008-2009) is spatially structured in a pattern largely stable between years (S. Lachish *et al.*, 2012), with the relative risk of infection by this parasite greater in the northern part of the study site and strongly associated with distance to the adjacent river Thames. In contrast, the relative risk *of P. relictum* infections was broadly uniform across the wood for the same avian hosts and time periods, with a significant cluster of infection occurring only in 2006 (S. Lachish *et al.*, 2012). These results are generally in accordance with the spatial prevalence patterns reported for breeding blue tits in 2001, 2003-2005 (Wood *et al*., 2007) and 2005-2008 (Knowles *et al*., 2011) (Figure 1.4), which analyzed data pooled for these time periods, but used less formal methods for analyzing spatial clustering of disease. The use of multi-state models to estimate transmission rates, showed that (for blue tits) these are higher nearby the river and stable between years for *P. circumflexum*; *for P. relictum*, transmission rates were not influenced by distance to the river, but varied between years (Lachish *et al*., 2011b).

Figure 1.4 - *Plasmodium circumflexum* and *P. relictum* interpolated prevalence maps for blue tits in Wytham Woods, constructed using inverse distance weighting. Areas of high prevalence are illustrated in red, with orange, yellow, green, pale blue and dark blue indicating areas of decreasing prevalence. Data was pooled for 2005-2008. Black dots within-wood are nestbox locations where prevalence data was obtained (figure reproduced from Knowles *et al*., 2011).



Seasonal variation in *Plasmodium* prevalence has also been found at the study site (Cosgrove *et al.*, 2008) (Figure 1.5), although this is pronounced only for *P. circumflexum*. This species shows a first prevalence peak in spring, which may be due to a relapse phenomenon as found in other studies (Applegate 1971; Schrader *et al.*, 2003), with a second peak occurring in autumn. *P. relictum* showed a more stable seasonal pattern of prevalence. The occurrence of such striking spatiotemporal patterns of prevalence and transmission provide an opportunity to look into their associations with mosquito ecology.

Figure 1.5 - Seasonal variation in predicted *Plasmodium* prevalence in blue tits for 2003-2005 pooled data in Wytham Woods. Final optimized models with a non-linear smoothed function of sampling date were used to provide predicted prevalence values over a range of hypothetical sampling dates. Also shown are timing of putative *Plasmodium* relapse (from Applegate *et al*. (1971), who conducted experimental work with English sparrows elsewhere) and timing of tit breeding season and fledging (in Wytham Woods, from Knowles, 2009) (figure adapted from Cosgrove *et al*., 2008).



Thesis outline and aims

In this thesis, I investigated avian host-malaria parasite-mosquito associations and the role of mosquito ecology in shaping these parasite systems at a fine-scale in a temperate woodland where avian malaria is endemic. This thesis starts with a general introduction (**this chapter**) presenting this field of research, the main study site (a mixed deciduous woodland) and what is known about the avian hosts, malaria parasites and mosquitoes of its avian malaria system, and the thesis outline and aims. In **Chapter 2**, I use molecular genetic methods (PCR and DNA sequencing) to investigate avian host-malaria parasite associations in wild caught British mosquitoes at the main study site and elsewhere in Great Britain. In **Chapter 3**, I assess the occurrence of spatial patterns of mosquito abundance at a local scale and its environmental predictors in a British woodland habitat where avian malaria is endemic (the main study site), using mosquito sampling methodologies and generalized linear modelling (GLM). In **Chapter 4**, I investigate the relative importance of mosquito density per host (putative vector availability) and landscape variables in shaping spatial patterns of *Plasmodium* prevalence at a local scale, by analyzing with GLM bird, mosquito and landscape data collected at the main study site. In **Chapter 5**, I assess the occurrence of seasonal patterns of mosquito abundance and its environmental predictors at the main study site, using GLM and mosquito sampling methodologies, while discussing its implications for the seasonal transmission of avian malaria. The thesis ends with a final discussion of the work presented here in the context of avian malaria and mosquito ecology research (**Chapter 6**).

I aimed to provide insights into the following research questions:

- Which mosquitoes in the British fauna are associating with avian malaria parasites and hosts in the wild? (Chapter 2)
- To what extent can mosquito abundance show significant spatial variation at a local scale in British woodland habitats, and what suites of environmental predictors may be driving it? (Chapter 3)
- What is the relative importance of mosquito density per host and landscape variables in shaping spatial variation in avian *Plasmodium* prevalence? (Chapter 4)
- To what extent do climatic variables (alongside calendar date) predict seasonal variation in mosquito abundance in British woodland habitats? What is the seasonal timing of avian malaria transmission in these habitats (Chapter 5)

Author contributions

Authorships and contributions for the data chapters in this thesis

	R. Alves	**M. J. Wood**	**B. V. Purse**	**S. Lachish**	**C. Cowell**	**I. Sepil**
Chapters co-authored	**2,3,4,5**	**2,3,4,5**	**3,5**	**4**	**3**	**4**
Contribution						
Writing of chapter, data analysis	2,3,4,5					
Mosquito sampling	2,3,4,5				3	
Blue tit sampling	4,5	4,5				
qPCR screening	4,5	4,5				4
Nested PCR screening	2					
Derived variables using GIS software	3,4	3,4				
Derived climatic variables	3,5					
Comments on drafts		2	3,5			
Guidance on analysis			3,5	4		
Intellectual input	2,3,4,5	2,3,4,5	3,5			

	S. M. Schäfer	**M. E. Taylor**	**S. C. L. Knowles**	**B. C. Sheldon**
Chapters co-authored	**2**	**5**	**4**	**2,3,4,5**
Contribution				
Mosquito sampling	2			
Blue tit sampling			4	
qPCR screening			4	
Climatic data		5		
Comments on drafts				2,3,4,5
Guidance on analysis				3,4,5
Intellectual input				2,3,4,5

Contribution of R. Alves to appendices

Appendix 1 - qPCR screening

Appendix 2 - qPCR screening, blue tit sampling and comments on drafts

Appendix 3 - qPCR screening, blue tit sampling and comments on drafts

Molecular analysis of avian *Plasmodium*-mosquito-host associations in British mosquitoes

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Abstract

Avian malaria has become an increasingly popular system for the study of parasite-host interactions. Knowledge regarding its vectors is essential to fully understand such systems due to their role in malaria parasite life cycle and transmission, but until very recently they have remained almost unstudied in the wild. In the present study, molecular genetic techniques were used to investigate avian *Plasmodium* and host associations in 12 British mosquito species or species groups, collected in woodland, agricultural and pasture habitats. The ornithophilic *Cx. pipiens/torrentium* group was found to be associated with four different avian *Plasmodium* lineages (maximum likelihood estimated proportion of infected individuals of 42.96 per 1000 in Somerset and 4.22 per 1000 in Wytham Woods,

Oxfordshire). No other mosquito species or species group was found associated with avian *Plasmodium*, suggesting this malaria parasite group can present high vector specificity and be tightly co-evolved with a given vector group. Several mosquito species or species groups were found to have fed on the blood of avian (and mammalian) host species, providing novel insights into the feeding preferences of British mosquitoes. Further studies looking into avian malaria in similar habitats should ideally attempt to comprehensively sample the occurring mosquito fauna, while focusing on ornithophilic specimens. To our knowledge, this is the first report of avian *Plasmodium* associations with wild mosquitoes in Great Britain.

Keywords

Avian malaria, *Plasmodium*, mosquito, blood meal preferences, wild populations

Introduction

Mosquitoes vector malaria, with the World Health Organization considering vector control as the most generally effective method to prevent human malaria transmission (WHO, 2006). The efficiency of malaria transmission is a function of vector abundance, distribution, host preference and feeding frequency, but is primarily a function of vector competence, i.e. the innate ability of a species to permit development of a given parasite for further transmission to a vertebrate host (MacDonald, 1957). Research on human malaria shows that even closely related species of mosquito may vary in vector competence (Gosh *et al*., 2000). Human *Plasmodium* is capable of development in only approximately 16% of

Anopheles species, with *Anopheles* the only other mosquito genera (N=110) able to transmit human malaria (Becker *et al.*, 2010). Therefore, vectors present ecological and physiological barriers for malaria parasite transmission among vertebrate host populations or different vertebrate host species, shaping parasite distribution with likely consequences for their evolution. This makes knowledge regarding mosquito-*Plasmodium* associations essential in understanding malaria prevalence and evolution, but in contrast with human malaria relatively little information exists regarding avian *Plasmodium* (Valkiūnas, 2005; Njabo *et al*., 2011). These parasites have played a historically important role as a model system to uncover human malaria life cycle, cultivation in vitro and chemical therapy, resulting in a wealth of knowledge about avian malaria vectors in laboratorial conditions, but with rather little work done in wild conditions (Valkiūnas, 2005 and references therein). Avian malaria has also become a popular model system to study parasite-host interactions in the wild (e.g. Bensch & Åkesson, 2003; Wood *et al*., 2007).

Avian malaria vectors only recently started being studied in the wild, with molecular techniques uncovering a higher avian *Plasmodium* diversity than the morphological methods previously used in the laboratory (e.g. Huijben *et al*., 2007; Gager *et al.*, 2008; Ishtiaq *et al.*, 2008; Kim & Tsuda, 2010; Kimura *et al.*, 2010; Carlson *et al*., 2011; Njabo *et al*., 2011). The studies have shown that not all sampled mosquito species are associated with avian *Plasmodium*, and that these malaria parasites occur at low prevalence (usually 1-5%, but may approach 10-30%). Most avian *Plasmodium* species have a cosmopolitan distribution and a broad avian host range (Valkiūnas, 2005; Bensch *et al*., 2009), suggesting they may be vector generalists. Alternatively, vector-malaria parasite co-evolution may result in high vector specificity, with broad parasite distribution reflecting broad vector

distribution. Our knowledge regarding vector specificity remains limited for avian *Plasmodium*, with recent work most often suggesting non-specific associations between avian malaria and mosquitoes in temperate and tropical systems (e.g. Ishtiaq *et al*., 2008; Kimura *et al.*, 2010; Njabo *et al*., 2011), though specific associations have also been found (Gager *et al*., 2008). Overall, avian *Plasmodium*-mosquito associations and specificity are little studied in Europe (but see Huijben *et al*., 2007; Glaizot *et al*., 2012; Ventim *et al*., 2012).

Great Britain is an island located in Europe, with a total of 31 mosquito species or species complexes recorded (Medlock *et al.*, 2005). From these, *Ae. vexans*, *Cx. pipiens*, *Cs. annulata*, *Cs. longiareolata*, *Cs. morsitans*, *Oc. communis*, *Oc. dorsalis* and *Da. geniculata* are proven vectors in experimental conditions of one or more avian *Plasmodium* species (Valkiūnas, 2005 and references therein). *Cx. pipiens* has been found associated with avian *Plasmodium* in the wild outside Great Britain (Huijben *et al*., 2007; Ejiri *et al*., 2009; Kim *et al*., 2009a; Hughes *et al*., 2010; Kim & Tsuda, 2010; Kimura *et al*., 2010; Ejiri *et al*., 2011a; Ejiri *et al*., 2011b; Glaizot *et al*., 2012; Inci *et al*., 2012; Ventim *et al*., 2012). The biology of a given mosquito species influences their putative associations with avian *Plasmodium*, with most mosquito species found in Great Britain capable to some extent of blood feeding on birds (Medlock *et al*., 2005 and references therein). Those that are ornithophilic (i.e. prefer to feed on birds) are more likely to play a preeminent role in transmission of avian pathogens, as higher rates of contact may facilitate co-evolution likely to render such mosquitoes more efficient vectors.

Here we screened mosquitoes collected in two different regions of southern Great Britain (the counties of Oxfordshire and Somerset) for *Plasmodium*, while blood fed mosquitoes were analyzed to assess host preferences, using molecular techniques. Collection sites included Wytham Woods (Oxford, United Kingdom), where *Plasmodium*-avian host associations have been unveiled (Cosgrove *et al*., 2008; Knowles *et al*., 2011). The aims of this study were to: (1) investigate malaria parasite-mosquito associations to identify potential vectors of avian *Plasmodium* in the collection areas and obtain insights into the degree of vector specificity exhibited by these malaria parasites; (2) examine how often the mosquito species feed on birds, an important trait for avian *Plasmodium* transmission. To our knowledge, this is the first study of avian *Plasmodium* associations with wild mosquitoes in Great Britain.

Methods

Mosquito collections

Collections were undertaken from April to September, as most mosquito species in Great Britain show limited or no adult activity in the winter months (Cranston *et al*., 1987; Medlock *et al*., 2005). Mosquitoes were collected in Wytham Woods (Oxford, United Kingdom; 51°46'N, 1°20'W), a mixed deciduous woodland, from 2008 to 2011. This woodland, and its surroundings, has wide availability of blood meal sources (e.g. deer, cattle, pheasants, wild birds), resting and breeding sites (Savill *et al*., 2010). CDC (Centers for Disease Control) incandescent light traps (Sudia & Chamberlain, 1961) Model 1012 and 512 (John W. Hock ©, Gainesville, United States of America) were used to sample

mosquitoes, with one of the Model 512 traps having as light source a fitted ultraviolet LED (Maplin©, Manvers, United Kingdom, 4 V, 420nm wavelength peak); traps were used approximately fortnightly from April to September, baited with CO_2 and placed 1.5-2m above ground. The same traps were placed in the tree canopy, 5-8 m above ground, to target ornithophilic specimens (Hutchinson *et al*., 2007; Russell & Hunter, 2011), fortnightly from June to September in 2011. During this period, gravid traps Model 1712 (John W. Hock ©, Gainesville, United States of America) were also used, to target blood-engorged females in search of an oviposition site (Reiter, 1983). Further sampling during this period was made with a sweep net (0.4m in diameter), by the ground vegetation and lower tree branches (less than 2m in height), to target resting blood-fed specimens (Kim & Tsuda, 2010). Collections were also made at a poultry farm within the John Krebs Field Station (Oxford, United Kingdom), located less than 500m from Wytham Woods, approximately fortnightly from May to September in 2010. CDC incandescent light traps model 1012 (John W. Hock ©) were placed 1.5-2.0m above ground, one meter from chicken (*Gallus gallus*) enclosures, aiming to target ornithophilic mosquitoes. Collections were also made at a broader geographical scale within a 540km^2 area in Somerset (United Kingdom) (51°01'N to 51°15'N; 02°39'W to 02°56'W), consisting mainly of pasture and agricultural land within river flood plains, with a wide availability of blood meal sources (e.g. cattle, wildfowl, starlings, waders). Miniature blacklight (UV) traps Model 1212 (John W. Hock ©, Gainesville, United States of America) were used, baited with CO_2 and placed 1.5-2m above ground, in tree patches on the edge of agricultural and pasture land, with collections undertaken usually monthly from June to September in 2008-2010. Occasional sampling was also undertaken within Oxfordshire (United Kingdom), in August 2008 at

Goring (51°31'N, 01°07'W), Aston (51° 43'N, 01°30'W) and Berinsfield (51°39'N, 01°10'W), with the trap type used in Somerset.

Extraction of DNA

Plasmodium is acquired in a female mosquito blood meal and enters the basal lamina of the mosquito gut, situated in the abdomen, to develop firstly into oocysts and afterwards into sporozoites. Sporozoites need to reach the mosquito salivary glands, situated in the thorax, for malaria parasite transmission. Therefore, female specimens were separated into thoracic (including the salivary glands) and abdominal parts, in order to obtain insights into the degree of development of the malaria parasites within their putative vectors. *Plasmodium* detection in unfed specimens, i.e. with no visible sign of a previous blood meal, is a strong indicator of malaria parasite development at least into ookinetes within a putative vector. Hence, body parts of unfed specimens were pooled (1-10 per pool) by type (abdomen or thorax), species (Cranston *et al*., 1987), collection method and collection site for further processing. Salivary glands were dissected from a subset of unfed specimens using standard mosquito dissection methods, as the presence of malaria parasites in these glands is a strong indicator that a mosquito is likely to be able to transmit the parasite (Njabo *et al*., 2009; Njabo *et al*., 2011). Body parts of engorged and gravid specimens were processed individually, as their blood feeding (which typically precedes egg development) renders then more likely to carry *Plasmodium*. Their pooling would therefore be more likely to mask the presence of *Plasmodium* strains in mixed infections within-pools than with unfed specimens. Furthermore, *Plasmodium* detection in engorged specimens may be due to their presence in the blood meal only. *Cx. pipiens* and *Cx. torrentium* cannot be differentiated

based on morphology alone with certainty (Becker *et al*., 2010), therefore specimens identified as either of these (Cranston *et al*., 1987) were considered as a *Cx. pipiens/torrentium* species group.

Genomic DNA of specimens collected from 2008 to 2010 in Wytham Woods and the nearby poultry farm was extracted using a standard ammonium acetate method (Bruford *et al*., 1998). For specimens collected elsewhere, the DNeasy Tissue Kit (Qiagen) was used following the modified procedures of Plichart *et al.* (2006) and Ishtiaq *et al.* (2008). Briefly, mosquito body parts were dried at 70 °C for two hours and crushed in 180 μl PBS using a sterile pestle tissue grinder (Fisher Scientific), with 200 μl of DNA extraction being obtained following the modified procedures of Plichart *et al*. (2006). Both extraction methods were tested on thorax and abdomen pools (1-10 specimens per pool) of *An. stephensi*, putatively infected by artificial feeding with *Plasmodium berghei*, a rodent malaria parasite, under laboratory conditions (frozen specimens kindly supplied by Johannes Dessens, London School of Hygiene and Tropical Medicine). All pools extracted using the ammonium acetate method (4 thorax and 4 abdomen) were later scored as positive for *Plasmodium* using a polymerase chain reaction (PCR) method (Waldenström *et al*., 2004). For the DNeasy Tissue kit, 7 out of 8 thorax and abdomen pools were scored as positive. The single negative pools were composed of a single thorax and abdomen belonging to the same mosquito specimen, which may have failed to blood feed. Therefore, both DNA extraction methods appear to yield extracts suitable for *Plasmodium* detection by PCR.

Plasmodium sp. detection

DNA extracts were screened for *Plasmodium* using a nested PCR (Waldenström *et al*., 2004) performed as described in Wood *et al*. (2007), to amplify a 478bp fragment from the *Plasmodium* cytochrome *b* gene. Positive (*Plasmodium relictum* DNA) and negative (ddH_20) controls were included in each PCR performed. PCR products were loaded into 2% agarose gel stained with ethidium bromide, electrophoresed and visualized under UV light. Samples showing visible amplification were purified using MinElute 96 UF PCR Purification Kits (Qiagen) and QiaVac Multiwell vacuum manifolds (Qiagen). Purified DNA fragments were sequenced directly using dye terminator cycle sequencing (BigDye version 3.1) and an ABI PRISM 310 automated sequencer (Applied Biosystems). Sequences were aligned and corrected by eye in Sequencher version 4.2 (Genecodes). Sequences unambiguously readable across 450 bp were compared with published sequences in the GenBank database (http://blast.ncbi.nlm.nih.gov) using a BLASTN search (using standard settings). Sequences with less than 100% identity to those in the database were checked by re-sequencing and assigned new names if they represented genuinely new sequences. *Plasmodium* positives from unfed mosquito samples were screened for avian blood (see below), to confirm that malaria parasite presence was not due to visually undetected blood meal remnants. The proportion of infected individuals in pooled samples was calculated with the bias-corrected maximum likelihood estimation (MLE) method with 95% confidence skewness corrected intervals (CI), using the program PooledInf Rate (Biggerstaff, 2006).

Blood meal analysis

DNA extracts from abdomens of visually detected blood fed mosquitoes (Sella scale: 2 to 6) were screened individually for host identification. Screening for avian blood was undertaken by PCR with primer pair 5′-GAC TGT GAC AAA ATC CCN TTC CA-3′ and 5′-GGT CTT CAT CTY HGG YTT ACA AGA C-3′, which amplifies a 508 bp fragment of the avian mitochondrial cytochrome *b* gene (Cicero & Johnson, 2001); positive (*Cyanistes caeruleus* DNA) and negative (ddH_20) controls were included in each PCR performed. Screening for mammalian, amphibian and reptilian blood was undertaken by PCR with primer pair 5′-CGA AGC TTG ATA TGA AAA ACC ATC GTT G-3′ and 5′-TGT AGT TRT CWG GGT CHC CTA-3′ (Molaei & Andreadis, 2006), which amplify a 772bp fragment of the cytochrome *b* gene (Ngo & Kramer, 2003); positive (*Bos taurus* DNA) and negative (ddH_20) controls were included in each PCR performed. Conditions for all PCR reactions were those of Molaei & Andreadis (2006), with the thermal profile according to Hamer *et al*. (2009). PCR products were treated as described above to obtain unambiguous sequences readable across 450bp for avian products, or 700bp for mammalian, amphibian or reptilian products. These were compared with published sequences in the GenBank database for host assignment, with all providing a match 99% identity (no gaps) or higher. Samples scored as positive for an avian host were screened for *Plasmodium* using the nested PCR reaction described above.

Results

Plasmodium detection

A total of 2759 unfed mosquitoes, from 12 different species or species groups, were screened for *Plasmodium*, in both their thoracic and abdominal parts (Table 2.1). Overall, *Plasmodium* was found in 24 abdomen pools of unfed *Cx. pipiens/torrentium*. The proportion of infected individuals by maximum likelihood estimation (MLE) is 30.33 per 1000 (95% CI 20.17-43.89), with 22 from Somerset (MLE=42.96 per 1000 (95% CI 28.13-62.24)), one from Wytham Woods (MLE=4.22 per 1000 (95% CI 0.24-20.34)); from specimens collected 1.5-2.0m above ground) and one from the nearby poultry farm (MLE=66.10 per 1000 (95% CI 3.83-306.81)). *Plasmodium* prevalence calculated by MLE was significantly higher in Somerset than in Wytham Woods for *Cx. pipiens/ torrentium* (Somerset=42.96/1000; Wytham=4.4/1000; Fisher's Exact Test, $P<0.001$; considering only June-September period when collections from both areas were available). Avian DNA was not detected in any *Plasmodium* positive sample. *Plasmodium* DNA was not detected in the thorax of any mosquitoes or directly from the salivary glands of 92 mosquitoes collected in Wytham Woods; species or species groups screened in the salivary glands were *Cs. annulata* (N=2), *Oc. cantans* (N=4), *Da. geniculata* (N=1), *Oc. annulipes* (N=71) and *Cx. pipiens*/*torrentium* (N=1).

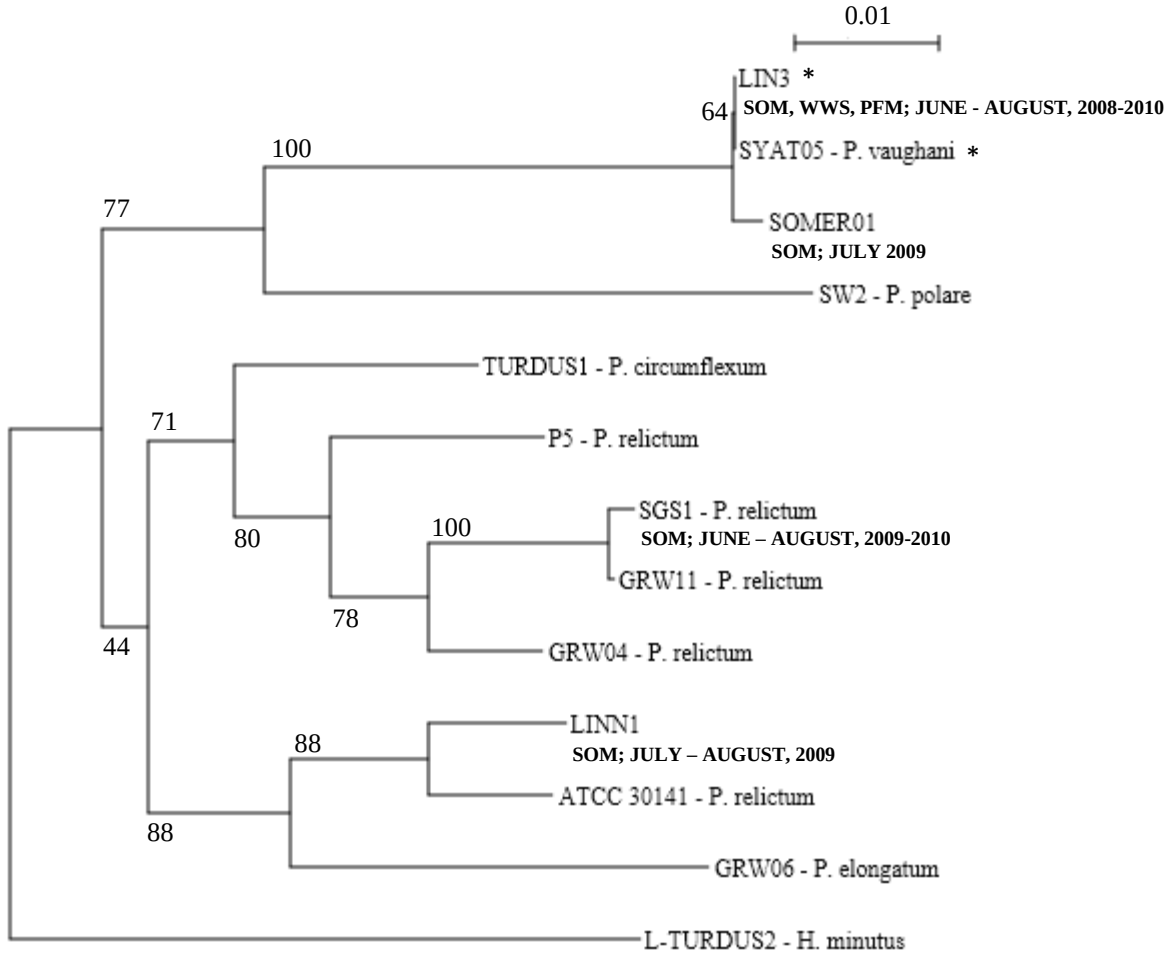
In mosquitoes from Somerset, 4 distinct *Plasmodium* lineages were found, with LIN3 representing 63.7% of the lineages recovered, followed by SGS1 (22.7%), LINN1 (9.1%) and a single previously unknown lineage, named SOMER01 (4.5%). LIN3 was the only lineage found in Wytham Woods and the nearby poultry farm. Figure 2.1 shows the

phylogenetic relationships between these lineages. SGS1 has been assigned to morphological species *Plasmodium relictum* (GenBank AF495571), with LIN3 (GenBank JN415758) and LINN1 (GenBank GQ471953) not assigned to a morphological species but considered as *Plasmodium* sp.; SOMER01 differs by a single nucleotide from lineage LIN3, and was therefore considered as *Plasmodium* sp. From lineages previously assigned to a morphospecies, LIN3 and SOMER01 share more similarity with SYAT05 (GenBank DQ847271; *P. vaughani*; 99% locus overlap and 100% similarity with LIN3; 99% locus overlap and 99% similarity with SOMER01) and LINN1 with ATCC 30141 (GenBank AY733089; 100% locus overlap, 98% similarity; *P. relictum*).

Blood meal analysis

A total of 102 blood-fed abdomens were screened for host identification (Table 2.2). This was successfully achieved for 37 specimens (36.3%), with 3 (8.1%) and 34 (91.9%) of positive samples scored as avian or mammalian respectively; no mixed blood meals were identified. Blackbird (*Turdus merula*) was assigned as avian host for one *Cx.pipiens*/*torrentium* collected in Somerset. Great tit (*Parus major*) was assigned as avian host for one *Cs. morsitans* and one *Culiseta* sp., both collected in Wytham Woods; such host assignments to species level are novel for British mosquitoes (Service, 1969; Service, 1971). *Plasmodium* was not found in any of the three blood-fed mosquitoes which had fed on avian hosts. Mammalian host assignments were given for 5 mosquito species or species complex: *An. claviger* (N=3), *An. maculipennis* complex (N=4), *Oc. annulipes* (N=63), *Oc. cantans* (N=1) and *Oc. rusticus* (N=22). The species *Cx. pipiens*/*torrentium* (N=1) and *Cs. morsitans*(N=2) had no assigned mammalian hosts. Novel host assignments to species level

Figure 2.1 - Phylogenetic tree of avian haemosporidian lineages found in *Cx. pipiens/torrentium*. The neighbor-joining method was used in SEAVIEW 4.3, with a Kimura 2-parameter distance matrix and lineage L-TURDUS2 (avian haemosporidian morphospecies *Haemoproteus minutus*) as the outgroup. Bootstrap support value is shown next to each branch. The scale bar indicates number of nucleotide substitutions per site. Geographical and temporal origin of lineages found in this study are shown under lineage name (SOM=Somerset, WWS=Wytham Woods, PFM=poultry farm nearby Wytham Woods). Other *Plasmodium* lineages were added for comparative purposes, including isolates from European mosquitoes elsewhere (*Cx. pipiens*: SYAT05, SW2, TURDUS1, P5, GRW11; *Cx. theileri*: SYAT05), isolates assigned to morphospecies most similar to lineages LIN3, SOMER01 and LINN1 (SYAT05 and ATCC 30141) and representative isolates found worldwide in avian hosts (GRW04 and GRW06).



* LIN3 and SYAT05 share 99% locus overlap, with 100% locus similarity in the overlapping region.

Table 2.1 - Number of specimens and pools processed for *Plasmodium* detection. "N" and "Pools" = number of specimens and pools, respectively, of a species or species group screened. "MLE" = maximum likelihood estimation (upper, lower 95% confidence skewness corrected intervals), per 1000 (‰) mosquitoes, based on pool size, of the proportion of infected individuals in pooled samples.

	Wytham Woods			**Poultry farm nearby Wytham Woods**		
	N	Pools (positive/total)	MLE (‰)	N	Pools (positive/total)	MLE (‰)
An maculipennis	2	(0/2)	0	29	(0/9)	0
An plumbeus	105	(0/37)	0	1	(0/1)	0
An. claviger	148	(0/41)	0	7	(0/2)	NA
Cq. richiardii	8	(0/6)	0	0	0	NA
Cs. annulata	91	(0/43)	0	10	(0/5)	0
Cs. morsitans	52	(0/19)	0	0	0	NA
Culiseta sp.	7	(0/4)	0	0	0	NA
Cx. pipiens/torrentium	238	(1/69)	4.22 (0.24-20.34)	16	(1/5)	66.10 (3.83-306.81)
Da. geniculata	73	(0/27)	0	0	0	NA
Oc. annulipes	942	(0/209)	0	0	0	NA
Oc. cantans	0	0	NA	0	0	0
Oc. rusticus	31	(0/17)	0	0	0	NA
Total	1697	(1/474)		63	(1/22)	
	Somerset			**Oxfordshire (other locations)**		
	N	Pools (positive/total)	MLE (‰)	N	Pools / (positive/total)	MLE (‰)
An maculipennis	25	(0/12)	0	0	0	NA
An plumbeus	0	0	NA	0	0	NA
An. claviger	39	(0/19)	0	0	0	NA
Cq. richiardii	137	(0/32)	0	0	0	NA
Cs. annulata	43	(0/20)	0	5	(0/3)	0
Cs. morsitans	8	(0/5)	0	0	0	NA
Culiseta sp.	0	0	NA	0	0	NA
Cx. pipiens/torrentium	580	(22/138)	42.96 (28.13-62.24)	39	(0/10)	0
Da. geniculata	0	0	NA	0	0	NA
Oc. annulipes	123	(0/27)	0	0	0	NA
Oc. cantans	0	0	NA	0	0	NA
Oc. rusticus	0	0	NA	0	0	NA
Total	955	(22/253)		44	(0/13)	

Table 2.2 - Number of blood-fed specimens screened for avian, mammalian, amphibian and reptilian blood meals, and host assignments. ”N” = number of specimens screened. “ID” = number of host assignments.

	Wytham Woods								Somerset		
	N	ID	Cattle	Roe deer	Fallow dear	Muntjac deer	Rabbit	Great tit	N	ID	Blackbird
An maculipennis	1	1	1	0	0	0	0		3	0	
An. claviger	2	1	1	0	0	0	0		1	0	
Cq. richiardii	0	0							1	0	
Cs. annulata	2	0							0	0	
CS. morsitans	2	0						1	0	0	
Culiseta sp.	1	0	0					1	0	0	
Cx. pipiens/torrentium	0	0							1	1	1
Da. geniculata	2	0							0	0	
Oc. annulipes	62	29	17	4	2	3	3		1	0	
Oc. cantans	1	1	1	0	0	0	0		0	0	
Oc. rusticus	22	2	2						0	0	
Total	95	34	22	4	2	3	3	2	7	1	1

regarding British mosquitoes are: cattle (*Bos taurus*) for *Oc. rusticus*, and fallow deer (*Dama dama*), roe deer (*Capreolus capreolus*), Muntjac deer (*Muntiacus reevesi*) and rabbit (*Oryctolagus cuniculus*) for *Oc. annulipes* (Service, 1969; Service, 1971).

Discussion

This study aimed to assess *Plasmodium* and host associations for mosquitoes collected in a previously studied avian malaria system (Wytham Woods) and other locations in Great Britain. Four different *Plasmodium* lineages (one of them previously assigned to the morphospecies *P. relictum*) were found associated with *Cx. pipiens*/*torrentium*, with the proportion of infected individuals, by maximum likelihood estimation, ranging from 4.22

(Wytham Woods) to 66.10 (poultry farm nearby Wytham Woods) per 1000. *Cx. pipiens*/*torrentium* and *Cs. morsitans* were shown to feed on avian hosts (blackbird and great tit, respectively).

Avian Plasmodium-mosquito associations

Only *Cx. pipiens/torrentium* was found associated with avian *Plasmodium*. This is in accordance with results from previous studies that found avian *Plasmodium*-mosquito associations restricted to a limited number of species (e.g. Gager *et al*., 2008; Ejiri *et al*., 2009; Kimura *et al*., 2010; Ejiri *et al*., 2011b). The apparent absence of avian *Plasmodium* in *Cs. annulata* (N=149), *Cs. morsitans* (N=160) and *Da. geniculata* (N=73), which have been associated with these parasites in laboratory studies (Valkiūnas, 2005 and references therein), indicates they may not have a role in transmission of avian *Plasmodium* in our study areas, or that the prevalence is so low that a higher number of specimens that had fed on birds is needed. Collections made using methodologies targeting ornithophilic (traps placed in the canopy and vicinity of a poultry farm) or previously engorged (sweep netting and gravid traps) mosquitoes yielded only 131 of the 2759 specimens screened for avian *Plasmodium*. Of these, only a single pool of *Cx.pipiens/torrentium* was positive, from specimens collected at the poultry farm. This is a farm site, while the avian hosts present (*Gallus gallus*) in the immediate vicinity of the traps are not natural hosts for the British mosquito fauna. The remaining 2628 specimens were collected with methodologies generally targeting host seeking females, with many likely to have recently emerged or be non-ornithophilic. The use of traps with a wild avian host, in more natural conditions, could

provide a higher yield of ornithophilic mosquitoes (e.g. Balenghien *et al*., 2006; Russell *et al*., 2011) and perhaps a higher estimate of prevalence for avian malaria.

The results here suggest that *Cx. pipiens* or the closely related *Cx. torrentium* may act as vectors for the avian malaria parasite lineages they were found associated with in the study areas. For *Cx. pipiens*, this assumption is consistent with its use in experimental avian *Plasmodium* laboratory infections (Valkiūnas 2005 and references therein; Vézilier *et al*., 2010). Associations between wild-caught *Cx. pipiens* and avian *Plasmodium* have also been reported elsewhere (Huijben *et al*., 2007; Ejiri *et al*., 2009; Kim *et al*., 2009a; Hughes *et al*., 2010; Kim & Tsuda, 2010; Kimura *et al*., 2010; Ejiri *et al*., 2011a; Ejiri *et al*., 2011b; Glaizot *et al*., 2012; Inci *et al*., 2012; Ventim *et al*., 2012), with parasite presence being confirmed within their thorax, where the salivary glands are situated (Kim & Tsuda, 2010; Inci *et al*., 2012; Glaizot *et al*., 2012). Lineages SGS1 (*Plasmodium relictum*) and LINN1 have been previously found elsewhere in wild *Cx. pipiens* (Kim & Tsuda, 2010; Kimura *et al*., 2010; Glaizot *et al*., 2012; Ventim *et al*., 2012). To our knowledge, no associations between *Cx. torrentium* and avian *Plasmodium* have been established. To fully establish vector competence, experimental infection of putative vectors is desirable (Ishtiaq *et al*., 2008; Njabo *et al*., 2011). Confirmed presence of the parasites in the salivary glands, situated in the thorax, is still a strong indicator of a transmission role by a given mosquito species (Njabo *et al*., 2009; Njabo *et al*., 2011). Both methods are logistically demanding and time consuming, requiring expertise and the possibility to maintain live specimens. Here, *Plasmodium* presence was confirmed in abdomen pools for *Cx. pipiens*/*torrentium* but not in thorax pools. Positive abdomen pools included only unfed specimens (none was positive for avian DNA), suggesting the malaria parasites were capable of developing in

this mosquito group, at least into ookinetes which are restricted to the gut. Taking into consideration the known vector role of *Cx. pipiens* elsewhere, it therefore seems reasonable that *Cx. pipiens/torrentium* may be the main vector transmitting the lineages found in the study areas.

The absence of *Plasmodium* positives in the thorax of *Cx. pipiens/torrentium* (222 pools screened) is unexpected. *Plasmodium* transmission in Somerset and Wytham Woods must necessarily be occurring, with at least some sporozoites reaching the salivary glands in the vector. Both DNA extraction methods used here were shown to yield thoracic and abdominal extracts suitable for malaria parasite detection in experimentally infected mosquitoes. Lower avian *Plasmodium* prevalence from thoracic as opposed to abdominal mosquito body parts has been reported for wild mosquitoes (Kim & Tsuda, 2010; Carlson *et al*., 2011), and such results are in accordance with the life cycle of avian *Plasmodium* within the vector, as sporogony is preceded by midgut infection. Sporogonic development of avian *Plasmodium* parasites is temperature dependent (Garnham, 1966; Valkiūnas, 2005). Oocyst diameter and sporozoite prevalence in salivary glands decrease significantly below a constant temperature of 21°C for *Plasmodium relictum* in *Cx. quinquefasciatus* (LaPointe *et al*., 2010), a sibling species of *Cx. pipiens*. Therefore, one hypothesis for the absence of *Plasmodium* positives in the thorax of *Cx. pipiens/torrentium* is the combination of low detection sensitivity of the methodology used with the exposure of avian *Plasmodium* found here to sub-optimal temperatures for development within the wild mosquitoes, resulting in numbers of sporozoites reaching the thoracic parts below detection threshold. Other hypothesis for this absence is the inability of the lineages found to develop into sporozoites in these mosquitoes, as arrested malaria parasite development at the

ookinete or oocyst stage can occur for a given mosquito-*Plasmodium* combination (Billingsley & Sinden, 1997). To our knowledge, *Cx. torrentium* has not been incriminated as a *Plasmodium* vector and the possibility this species largely composes the *Cx. pipiens/ torrentium* collected cannot be discarded. Hence, the *Plasmodium* lineages found here may arrest their development at ookinete or oocyst stage because their mosquito host is *Cx. torrentium*. Contrary to this hypothesis, *Cx. pipiens* is known to be abundant in Great Britain (Cranston *et al*., 1987; Medlock *et al*., 2005), while lineage SGS1 (*Plasmodium relictum*) has been used for experimental infections with this mosquito species (Vézilier *et al*., 2010) and was found in the thoracic parts of wild *Cx. pipiens* specimens (Kim & Tsuda, 2010). Therefore, it seems likely this lineage is able to develop into sporozoites in any *Cx. pipiens* collected here. The other lineages found here are yet to be found separately in *Cx. pipiens* thoracic body parts (LINN1), or associated with a mosquito species elsewhere (LIN3 and S0MER01). Overall, low PCR sensitivity is likely to be the main reason for absence of *Plasmodium* positives in thoracic pools. Therefore, formal testing of the detection threshold for methodology used is desirable in any future work. Experimental infections, with both mosquito species and the *Plasmodium* lineages found, are needed to fully establish the vector competence suggested by the results here.

Avian *Plasmodium* prevalence in *Cx. pipiens/torrentium* was low (MLE range: 4.22 to 66.10 per 1000). This may be due to unsuitability of the sampling methodology used, but nevertheless is in agreement with results for *Cx. pipiens* in other European regions, where reported infection rates for individually processed mosquitoes range between 1.7% and 6.6% (Huijben *et al*., 2007; Glaizot *et al*., 2012), while an analysis of pooled specimens yielded a minimun infection rate (number of pools positive for *Plasmodium* / number of

mosquitoes screened) of 0.35% (Ventim *et al*., 2012). Furthermore, it also agrees with the typical proportions of wild mosquitoes found infected with avian *Plasmodium* worldwide (Chapter 1, Table 1.1), including in studies using traps baited with avian hosts (Njabo *et al*., 2009; Kimura *et al*., 2010; Njabo *et al*., 2011). Hence, in Great Britain, as elsewhere, the occurrence of low *Plasmodium* prevalence in mosquitoes, even in known vector species, demands extensive mosquito sampling effort to obtain informative data.

There was a significant difference in *Plasmodium* prevalence between Somerset and Wytham Woods. The Somerset area consists mainly of pasture and agricultural land, with mosquito collections undertaken in patches of trees, while in Wytham Woods collections were undertaken within extensive woodland which provides higher availability of vegetation cover and resting sites. Tree patches may act as clustering sites in Somerset, increasing bird-mosquito contact and rendering these sites as hot-spots for parasite transmission, leading to nearby increased parasite prevalence in avian and mosquito hosts. In accordance with this possibility, West Nile virus prevalence in mosquitoes is higher in communal avian roosting sites (Diuk-Wasser *et al*., 2010) and bird mortality due to this virus has been reported to be higher in areas of lower forest cover (LaDeau *et al*., 2011). Landscape composition may therefore be a factor shaping avian parasite prevalence in mosquito vectors.

Lineage LIN3 was found in Wytham Woods and the nearby poultry farm in mosquitoes, but not in avian hosts sampled to date in the study site (Wood *et al*., 2007; Cosgrove *et al*., 2008). This highlights how avian host sampling may fail to uncover fully the parasite diversity present. Bird sampling is commonly done with mist nets or at small nestboxes, rendering it biased towards smaller and common passerine birds, missing larger birds or

birds preferentially inhabiting the canopy. In accordance with other studies assessing mosquito-avian *Plasmodium* associations (Kimura *et al*., 2010; Njabo *et al*., 2011), our results also suggest bird sampling needs to be more comprehensive whilst using diverse capture methods, to further uncover parasite diversity in avian communities.

Host-mosquito associations

Blood meal analysis showed that *Cs. morsitans* and *Cx. pipiens/torrentium* fed on the avian hosts great tit and blackbird (Table 2.2), in accordance with their known host preferences in Great Britain (Service, 1969; Service, 1971). This indicates that these species are possible vectors of *Plasmodium* in these avian hosts. *Cs. morsitans* fed in Wytham Woods on great tit (*Parus major*), an avian host known to carry *P. circumflexum* in this study site (S. Lachish *et al*., 2012). This mosquito species is a proven experimental vector of *P. circumflexum* (Meyer & Bennett, 1976), and the avian host association revealed here further indicates a possible role in *Plasmodium* transmission in Wytham Woods. In Somerset, 4 distinct *Plasmodium* lineages were retrieved from *Cx. pipiens/torrentium*, which were found to have fed on blackbird at this site. LINN1 has previously been found in a blackbird in France (Bentz *et al*., 2006) and also in New Zealand (Howe *et al*., 2011). Therefore, this host association suggests *Cx. pipiens/torrentium* may be transmitting lineage LINN1 to blackbirds in Somerset.

Further host assignments were all mammalian, in accordance with the known limited or absent ornithophily of British specimens of these five species or species complexes (*An. claviger, An. maculipennis* complex, *Oc. annulipes, Oc. cantans* and *Oc. rusticus*) (Service, 1971; Cranston *et al*., 1987). Host assignments by blood meal analysis were relatively few

in number and mostly mammalian. These assignments provide some novel insights into feeding habits of British mosquitoes, but they did not allow for strong insights into their ornithophily. The majority of blood-fed specimens (92 out of 102) were obtained by sweep net at ground level. This method may target resting ornithophilic mosquitoes poorly, as these may be more abundant near the canopy (Russell & Hunter 2005; Hutchinson *et al*., 2007). Blood meal digestion prior to sampling may have reduced the amount of DNA available for host assignment in the specimens screened, reducing the number of assignments accomplished (Kent, 2009).

Implications for avian malaria ecology and evolution

Avian *Plasmodium* was found in *Cx. pipiens/torrentium* only, in both Somerset and Oxfordshire. This is in accordance with Huijben *et al*. (2007) and Glaizot *et al*. (2012), who only found *Cx. pipiens* associated with avian malaria parasites in European habitats. A wide range of avian *Plasmodium* species are known to complete their development in *Cx. pipiens* (Valkiūnas, 2005 and references therein), or have been found associated with this mosquito in the wild. Therefore, one hypothesis is that avian *Plasmodium* transmission in Great Britain may be mediated mainly by this mosquito group. In accordance with this hypothesis, *Cx. pipiens* and *Cx. torrentium* are ornithophilic, explore a wide range of breeding habitats and are abundant and widely distributed in the whole and the southern part of Great Britain, respectively, (Cranston *et al*., 1987; Medlock *et al*., 2005), suggesting they may provide avian malaria with ample transmission opportunities. Our results thus suggest that avian *Plasmodium* may present high vector specificity, with ample vector distribution allowing for a putative extensive parasite distribution. Assessment of

Plasmodium prevalence in avian hosts in areas geographically close to our study sites, but void of *Cx. pipiens*/*torrentium*, would be informative about this possibility. High vector specificity suggests tight malaria parasite-vector co-evolution, with human *Plasmodium* known to vary in developmental success even in closely related vector species (Gosh *et al*., 2000). A similar scenario may be occurring between avian *Plasmodium* and *Cx. pipiens*/*torrentium*, in European habitats. This may be aided by a relatively sparser distribution, or lower abundance, of other possible vectors (such as *Cs. morsitans* or *Cs. annulata*) compared to other regions (Ishtiaq *et al*., 2008; Njabo *et al*., 2011), resulting in relatively more frequent encounters of *Cx. pipiens*/*torrentium* with avian hosts and malaria parasites. In this scenario, a higher adaptation of these parasites to this mosquito group would benefit transmission. Co-evolution can hypothetically be expressed in the vector by lower fitness costs from ingesting an infected blood meal (Ferguson *et al*., 2003) or a parasite-mediated increase in feeding activity (Schwartz & Koella, 2001). Assessing the fitness and behavioral effects of avian *Plasmodium* infection in mosquito species found here would aid in understanding if co-evolution has occurred between mosquito and parasite.

Conclusion

This study uncovered associations between mosquitoes, avian *Plasmodium* and avian hosts in the wild in Great Britain, with a limited number of associations being found. This agrees with results in other malaria systems, strongly suggesting that transmission of these parasites is being undertaken by only a subset of the overall mosquito fauna present. In

particular, the ornithophilic species *Cx. pipiens*/*torrentium* is likely to play a key role here, in accordance with their known vector status elsewhere in the wild. Avian *Plasmodium* may therefore present tight co-evolution and high specificity with a given vector species group in British habitats. Only ornithophilic specimens (from the above species group) were found associated with avian *Plasmodium*, suggesting that, as expected, these are more likely to have a key role in the transmission of avian malaria parasites in the study areas. This illustrates the value of targeted approaches to sample ornithophilic mosquitoes, in order to increase the existing knowledge regarding avian malaria systems in the wild. Nevertheless, a possible role of mosquito species that do not feed mainly on birds in transmitting these malaria parasites cannot be discarded, as high abundances and sympatry with avian hosts may result in the establishment of parasite-vector associations. Further studies on wild malaria systems concerning the British mosquito fauna should attempt to be comprehensive, while taking into consideration that ornithophilic mosquitoes are more likely to vector avian *Plasmodium*.

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Local-scale spatial patterns of mosquito abundance in a British woodland and their relationship with environmental variables

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Abstract

Spatial heterogeneity in mosquito (Diptera: Culicidae) abundance has been studied mostly in humanized habitats. Such variation may also occur in less altered natural habitats such as British woodlands, where the spatial prevalence of avian malaria (a mosquito borne disease) can vary at a fine spatial scale. In this study, local-scale spatial abundance patterns of adult mosquito species or species groups were assessed between June and September 2009 across 36 sampling points in a single woodland (385 ha) in Great Britain, where avian malaria is known to be endemic. Associations between abundance and environmental variables were evaluated using generalized linear modelling and Bayesian model averaging.

The analysis revealed non-random spatial patterns and significant environmental predictors for mosquito abundance, which varied between species or species groups. Location, micro-climate, and breeding site variables were associated with mosquito abundance for three of the mosquito species (*Oc. annulipes*, *An. plumbeus* and *Cs. annulata*). With the exception of *Oc. annulipes*, where a remarkable 90.8% of the deviance was explained by the model, the environmental variables considered had low explanatory power for mosquito abundance: 18.7 and 18.4 explained deviance for *Cs. annulata* and *An. plumbeus* respectively. This suggests abundance is being driven by a complex set of predictors. The results suggest that the study of British woodland mosquito populations and mosquito-borne disease, such as avian malaria, will benefit from considering the occurrence of local-scale spatial heterogeneity in mosquito abundance and the environmental variables shaping it.

Keywords

Culicidae, local-scale, spatial heterogeneity, environmental predictors, temperate woodland, avian malaria

Introduction

Vector-borne disease is responsible for high morbidity and mortality in human and animal populations (Valkiūnas, 2005; WHO, 2007; WHO, 2008), with their epidemiology a result of complex vector-host-parasite interactions under the influence of environmental variables, subject to climate change and habitat fragmentation (Tabachnick, 2010). Knowledge regarding the causes of spatial variation in vector-borne disease prevalence in wild animal

populations remains rather limited. Infection probability by a given parasite at a given location will depend on host-specific biotic variables such as host age, sex, immunity and population density (Keymer & Anderson, 1979; Poulin, 1996; Wilson *et al*., 2001; Palacios *et al*., 2007), but also on vector availability and behavior (Rogers & Randolph, 2006). The abundance and distribution of arthropod vectors such as mosquitoes is likely to be limited compared to hosts, due to lower mobility or a more restricted environmental range. Geomorphology and climate combine to form ecosystem structure, which vary in their capacity to support mosquitoes (Reisen, 2010). Landscape anthropogenic variables (e.g. artificial breeding sites such as containers or dams), may also alter mosquito availability (Reisen *et al*., 1990; Barker *et al*., 2009). Therefore, mosquito abundance and distribution is influenced by both landscape and climate, with these likely to differ between habitats and geographical scales.

Due to the medical importance of diseases such as malaria and dengue, the spatial relationship between mosquito vectors and disease prevalence in human host populations has been intensively studied in humanized environments (e.g. Trape *et al*., 1992; Thomson *et al.*, 1994; Ghebreyesus *et al.*, 1999; Clarke *et al.*, 2002; Diuk-Wasser *et al.*, 2005; Galardo *et al.*, 2009). Crucial in explaining this relationship is an understanding of the environmental variables influencing mosquito distribution and abundance. For example, breeding site location has been shown to influence mosquito abundance and thus to contribute to spatial variation in malaria, demonstrating the influence environmental variables can have on disease prevalence (Trape *et al.*, 1992; Clarke *et al.*, 2002). Pioneering studies looking into the relationship between mosquito distribution and/or abundance and avian malaria prevalence have yielded mixed results, but overall highlight

the need to consider and understand vector ecology when looking into prevalence patterns (van Riper *et al*., 1986; Woodworth *et al.,* 2005; Tompkins & Gleason, 2006; Ventim *et al*., 2012).

This study was undertaken under the scope of current research on host-parasite-vector interactions and the epidemiology of avian malaria (*Plasmodium* spp., *sensu* Valkiūnas *et al*., 2005) within a woodland site in South England (Wytham Woods, Oxford, United Kingdom). Here, pronounced spatial differences occur at a local scale for two breeding sympatric woodland tit (Paridae) populations, in the distribution of two avian malaria parasite species, *Plasmodium relictum* and *P. circumflexum* (Wood *et al.,* 2007; Knowles *et al.,* 2011; S. Lachish *et al*., 2012). Landscape features putatively associated with mosquitoes have been suggested to be the main determinants of infection risk and transmission for *P. circumflexum* (Lachish *et al*., 2011b; S. Lachish *et al*., 2012), highlighting possible local-scale spatial heterogeneity in mosquito abundance. In contrast, *P. relictum* prevalence suggests random patterns of mosquito distribution. This study aimed to (1) assess the occurrence of local-scale heterogeneity in adult mosquito abundance in a British woodland where avian malaria is endemic (Wytham Woods), and (2) explore species-specific associations with different suites of environmental variables including microclimate, canopy and potential breeding site location and evaluate whether these are consistent with known ecological characteristics of individual mosquito species or species groups. Such findings could aid in the planning of effective mosquito surveys and in the study of mosquito-borne disease of wild host populations in these habitats.

Methods

Study site

Wytham Woods is a mixed deciduous woodland (385ha) near Oxford, United Kingdom (51°46'N, 1°20'W). The site is situated on a loop of the River Thames which closely borders part of its northern limits, with the majority of the immediately surrounding areas being agricultural and pasture land. This woodland and its surroundings have wide availability of resting and breeding sites, and blood meal sources (e.g. deer, cattle, pheasants, blue tits), part of which has been the subject of studies regarding long-term monitoring of environmental changes and ecological processes (Savill *et al*., 2010).

Mosquito data

In the UK, adult mosquito abundance is generally very low for most species from October to April. Seasonal patterns of abundance are species-specific (Cranston *et al.*, 1987 and references therein; Medlock *et al.*, 2005 and references therein), making a sampling strategy that encompasses different seasonal periods more likely to uncover the spatial distribution of a larger number of species. Mosquito collections were therefore undertaken in three different sampling periods, between 1-5th June, 25-29th July and 9-12th September in a single year (2009). Miniature blacklight (UV) traps Model 1212 (John W. Hock ©, Gainesville, United States of America), baited with CO_2, were used for the collections, being placed at the sampling points for a 15-20 hour period encompassing at least 2 hours before and after sunset and sunrise. The known within-wood spatial variation in avian *Plasmodium* prevalence (see Introduction, Figure 1.4) signaled the possibility of spatial

variation in mosquito abundance throughout Wytham Woods; to encompass it, a constrained randomized design was used to allow for the selection of sampling points throughout the whole study site. From 61 locations spaced regularly in a 250m grid throughout Wytham Woods, four sampling points were randomly selected for sampling in each of nine approximately equally-sized geographical sub-areas of the wood (Figure 3.1).

Figure 3.1 - Temperate woodland study site and surrounding area, with location of sampling points, River Thames, and mapped potential breeding sites. Partition of study site in major and sub-geographical areas for sampling point selection (see Methods) is shown.

The same 36 points were used in the 3 sampling periods. Due to logistical constraints (the number of traps available), only the sampling points in 3 sub-areas were used daily (12 in total), thus requiring 3 collection nights to sample all 9 sub-areas. A randomized (without reposition) design was used to minimize spatial clustering of the sub-areas on a given collection night, with the study site being divided into three major geographic areas (Figure 3.1) and one sub-area within each being selected daily. Collections within each sampling period were carried out in days of expected low wind speed (<15 km/h) and no rainfall. In order to assess repeatability of the methodology used, an extra sampling effort was undertaken during 1-5th June, using the same sampling points of a previous collection night. Collected mosquito specimens were morphologically identified following Cranston *et al.* (1987) or classed as unidentified (due to loss of diagnostic features or uncertain diagnostic). Morphologic differentiation between *Cx. pipiens* and *Cx. torrentium* cannot be made with certainty (Becker *et al.,* 2010) and therefore specimens identified as either of these (Cranston *et al.,* 1987) were considered as a *Cx. pipiens/torrentium* species group.

A ground-based survey of potential mosquito breeding sites, classified as containers (artificial and without permanent vegetation and water) or ponds (natural or semi-natural with permanent water and vegetation), was undertaken between April and August 2009, inside the study site and on its surrounding area inside a 1km radius from the edge of the wood. Though they may be important breeding sites, temporary natural or semi-natural water bodies were not mapped due to their transient nature. Mosquito larvae and pupae were also occasionally collected from breeding sites in the study area, including water-filled tree-holes (not mapped) and the River Thames adjacent river beds and low current branches (see below). Identification of a subset of collected specimens, either at the larval

stage or after adult emergence (Cranston *et al.*, 1987), provided insights about breeding site use for several species or species groups at the study area (Table 3.1).

Environmental and landscape data

A GIS of the study site has been previously constructed, providing an accurate determination of the latitude and longitude of each sampling point (Wilkin *et al*., 2007). Collections with CDC light traps baited with CO_2 are considered to reflect mosquito abundance in its nearby vicinity, with a collection range smaller than 30m (Odetoyinbo, 1969; Service, 1993). Furthermore, mosquitoes show limited dispersal, with appetential flight being the norm and typically lasting until encountering a needed resource (resting and breeding sites, mates, nectar and blood meals), covering less than a few hundred meters (Service *et al*., 1997 and references therein). The study site is a relatively wind-sheltered habitat, where mosquito biological needs are expected to be generally provided within and in its close vicinity. Therefore, considering mosquito captures as a measure of their abundance at a given sampling point seems justified.

Mosquito life history parameters are influenced by temperature and humidity (Clements, 1992), making within-site differences in microclimatic conditions possible environmental drivers of mosquito abundance. Temperature (°C) and humidity (%RH) were recorded hourly at each sampling point location using DS1923 Temperature/Humidity logger ibutton® devices, from 0000 GMT April 10 to 0000 GMT September 13 2009. All hourly values for this period were pooled and the average calculated for each sampling point location to obtain average temperature (AT) and average humidity (AH). Canopy height can affect mosquito habitat quality by influencing host, breeding and resting site

availability (Clements, 1999). Average canopy height (ACH) in a 30m radius buffer around each sampling point was known (Hill *et al*., 2004).

Further possible environmental drivers of spatial variation in mosquito abundance at the sampling points were derived using GIS software (MapInfo Professional version 10.0):

(i) Distance (m) to the River Thames (DRT): river beds and low current branches can act as important mosquito breeding sites, with inverse relationships between adult abundance and distance to rivers previously described in tropical and semiarid habitats (Trape *et al.*, 1992; Cano *et al.*, 2006; Barker *et al*., 2009).

(ii) Average distance (m) to containers (ADC) and ponds (ADP): ponds and containers are preferred differently by different mosquito species as breeding sites (Cranston *et al*., 1987). To account for the possible cumulative effect of multiple potential breeding sites in shaping mosquito abundance, average distance to all mapped ponds (n=9) and average distance to all mapped containers (n=81) were derived separately.

Statistical analysis

Repeatability of the sampling method for assessing abundance levels was investigated by species-specific Pearson's product-moment correlation coefficient (r) tests, on collections made 3 days apart in the same 12 sampling points, between 1-5th June (an extra collection night was undertaken). These were significantly correlated ($r=0.75$, $P<0.001$), when considering the six most abundant species together (corresponding to a total of 311 specimens, comprising 89% of identifiable specimens here). Looking into within-species correlations here, *Oc. annulipes* (N=220) collections (71% of the identifiable total) were also significantly positively associated ($r=0.71$, $P<0.010$). For the remaining 5 species

(which comprised 29% of the identifiable collections here), associations were not significant (P>0.05) for each, but sample sizes were not as large as for *Oc. annulipes*. Hence, the sampling methodology used probably provides a reasonable way to assess mosquito abundance.

In accordance with expected species-specific differences in seasonal patterns of mosquito abundance, collections obtained for most species or species groups were very low for given sampling periods. Analysis of temporally pooled mosquito collections was therefore considered to allow for a more robust and informative spatial analysis of abundance at the study site. Hence, the sum of adults collected across the three sampling periods, for each species or species group, was calculated for each sampling point (n=36), and used as the dependent variable (henceforth mosquito abundance) in generalized linear models (GLM) (McCullagh & Nelder, 1989). For the 12 points sampled twice during 1-5th June, one of the sampling days was chosen at random for the analysis. Full GLMs containing two independent variables, latitude and longitude, were constructed to investigate species-specific spatial patterns of mosquito abundance within-wood. Other full GLMs were constructed to explore species-specific associations of mosquito abundance with six environmental variables: average canopy height, average temperature, average humidity, distance to the River Thames, average distance to all ponds and average distance to all containers. We expected different error structures to be appropriate between species or species groups. Therefore, each full model was fitted with Gaussian (using log transformed dependent variables), quasi-Poisson and negative binomial error structures, with the more appropriate for each species or species groups selected by visual examination of plotted deviance residuals against fitted values (Zuur *et al.*, 2009) (see Appendix IV for an example

of plotted deviance residuals against fitted values for a full model with different error structures). Collinearity between independent variables was assessed using variance inflation factors (VIF), for both starting and final models. Highly correlated variables for which VIF exceeded 5 were successively removed from the full model until VIF for all variables were below 5 (no variable presented VIF above 5 in the final models). Starting models were optimized by variable selection by Bayesian model averaging (BMA) (Raftery *et al*., 1995), using the BMA package (Raftery *et al*., 2005) for R 2.12.1 (http://www.R-project.org). This methodology averages over competing predictor combinations, accounting for the uncertainty inherent in the model selection process. The optimization output provides the probability of a predictor coefficient not being zero. Predictors with a probability higher than 90(out of 100) are mentioned here as strong predictors, and were always included in the optimized best-supported model for a given species or species group. Optimized best supported models also included predictors with a probability between 50 and 90 (out of 100) and with coefficient±standard deviation not spanning zero, mentioned here as moderate predictors. We hypothesized that mosquito abundance may decrease non-linearly with increasing distance from breeding sites and therefore linear distance variables in optimized best-supported models were substituted for quadratic terms, being retained if causing a significant decrease in deviance. For the final, best-supported, models the following were assessed: (i) percentage explained deviance [100 x (1 – residual deviance/null deviance)], as a measure of explanatory power for mosquito abundance (analogous to r^2) (Zuur *et al*., 2009); (ii) deviance residual patterns plotted against fitted values for model validation (Zuur *et al.*, 2009); (iii) presence of residual spatial autocorrelation (RSA), through visual examination of residual binned semivariograms

against a simulation envelope obtained from 999 Monte Carlo data permutations, using the geoR package (Pfeiffer *et al*., 2008) in R. No final model showed presence of RSA.

Results

A total of 1028 adult mosquitoes were collected at the study site (including the extra sampling effort on 1-5th June), with the six most abundant identified species or species groups being *Oc. annulipes*, *An. plumbeus*, *Cx. pipiens*/*torrentium*, *Cs. annulata*, *An. claviger* and *Da. geniculata* (Table 3.1), which together comprised 84% of the total collections and 94% of total identifiable specimens. GLM analysis was pursued for these species only.

Table 3.1 - Total number of adult mosquitoes collected ("N") and percentage of total catch for each species or species group ("%"), alongside confirmed breeding site use in the study area. Includes specimens collected during the extra sampling effort on 1-5th June.

Species or species group	N	%	Confirmed breeding site use
An. claviger	49	4.8	Ponds, low current branches (river Thames)
An. maculipennis complex	1	20.0	
An. plumbeus	206	<1	
Cq. richiardii	9	<1	
Cs. annulata	78	7.6	Ponds and temporary water bodies
Cx. pipiens/torrentium	122	11.9	Artificial containers , temporary water bodies
Da. geniculata	41	4.0	Water-filled tree holes
Oc. annulipes	374	36.4	Temporary water bodies
Oc. cantans	17	1.7	
Oc. rusticus	32	3.1	
Unidentifiable	99	9.6	
Total	1028		

Breeding site use recorded for several of these species at the study area (Table 3.1) was in accordance with previous findings for British mosquitoes (Cranston *et al*., 1987 and references therein; Medlock *et al*., 2005 and references therein).

Probably due to the large proportion of zero values, regardless of error structure GLMs showed substantial lack of fit for *An. claviger* and *Da. geniculata*, and therefore these species were not considered further here. Looking into local spatial patterns of mosquito abundance with successfully fitted GLMs, latitude and longitude were strong and moderate predictors, respectively, for *Oc. annulipes* (most abundant species), with these being more abundant in the north and west of the study site; latitude was a strong predictor for *Cs. annulata* (fourth most abundant species), with these being more abundant in the north (Table 3.2). Furthermore, testing for the effect of geographical sub-area in the mosquito abundance of the species or species groups successfully fitted with GLMs revealed a significant effect for *Oc. annulipes* (analysis of deviance: LR=58.06, P<0.001, negative binomial error structure) and *Cs. annulata* (F=3.28, P=0.010, quasi-Poisson error structure), but not for *Cx. pipiens/torrentium* (LR=14.39, P=0.072, negative binomial error structure) and *An. plumbeus* (F=1.26, P=0.305, Gaussian error structure). This is in accordance with the BMA outputs, providing further evidence of the occurrence of non-random spatial patterns of mosquito abundance within the woodland study site, which differ between species, but also that random patterns occur in other sympatric species or species groups.

Looking into associations with suites of environmental variables (Table 3.2), *Oc. annulipes* abundance was strongly negatively predicted by distance to the River Thames (Figure 3.2) and average distance to all ponds and moderately positively predicted by average temperature and average humidity; the best-supported model had a percentage explained

deviance of 90.8%, suggesting high explanatory power for *Oc. annulipes* abundance at the study site. For *An. plumbeus*, average humidity was a moderate positive predictor, with the best supported model having a percentage explained deviance of 18.4%, suggesting a low explanatory power. For *Cs. annulata*, average distance to all ponds was a moderate negative predictor (Figure 3.2), with the best-supported model having a percentage explained deviance of 18.7%, also suggesting low explanatory power. Quadratic terms of distance to breeding sites did not improve the best-supported models, suggesting linear relationships between these and the abundance of *Oc. annulipes* and *Cs. annulata* at the study site.

Figure 3.2 – Relationship between (a) *Oc. annulipes* abundance and distance to the river Thames (m), and (b) *Cs. annulata* abundance and average distance to all ponds (m) at the study site.

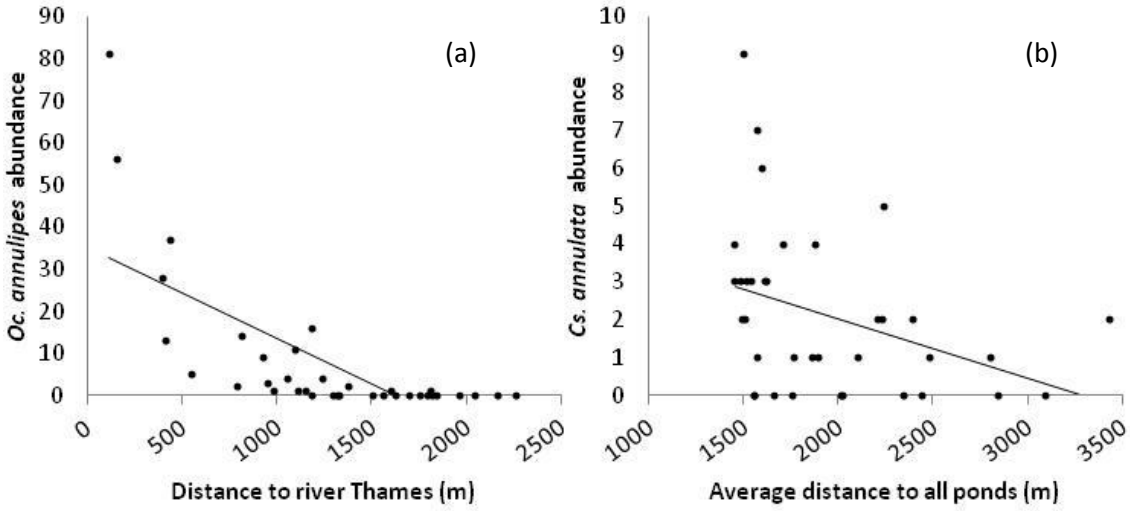

Table 3.2 - Statistical modelling of mosquito abundance at the study site.

	Oc. annulipes			*An. plumbeus*		
	Pr	**EV**	**± SD**	**Pr**	**EV**	**± SD**
Latitude	**100.0**	**0.0026**	± **0.00024**	29.9	0.000058	± 0.00012
Longitude	**93.6**	**-0.00070**	± **0.00032**	22.5	-0.000027	± 0.000076
Explained deviance (%)		90.79			0	
Error structure		Negative binomial			Gaussian	
Average canopy height (m)	57.6	-0.049	± 0.053	33.8	0.11	± 0.21
Average temperature (°C)	**74.1**	**1.77**	± **1.37**	31.7	2.81	± 5.29
Average humidity (%RH)	**71.3**	**0.22**	± **0.19**	**64.6**	**0.89**	± **0.87**
Distance to the river Thames (m)	**100**	**-0.0017**	± **0.00053**			
Average distance to all ponds (m)	**100**	**-0.0041**	± **0.0015**	25.7	-0.00077	± 0.0019
Average distance to all containers (m)				27.9	0.0019	± 0.0045
Explained deviance (%)		90.77			18.38	
Error structure		Negative binomial			Gaussian	
	Cx. pipiens/torrentium			*Cs. annulata*		
	Pr	**EV**	**± SD**	**Pr**	**EV**	**± SD**
Latitude	32.7	-0.00010	± 0.000019	**95.8**	**0.00061**	± **0.00024**
Longitude	34.8	-0.000089	± 0.00016	14.3	0.000010	± 0.000086
Explained deviance (%)		0			22.4	
Error structure		Negative binomial			Quasi-Poisson	
Average canopy height (m)	9.1	0.00072	± 0.012	22.4	0.0096	± 0.025
Average temperature (°C)	9.0	0.0072	± 0.23	13.3	0.069	± 0.35
Average humidity (%RH)	10.1	0.0051	± 0.035	23.8	0.032	± 0.077
Distance to the river Thames (m)				17.1	-0.000042	± 0.00019
Average distance to all ponds (m)	8.9	-0.00000024	± 0.00010	**90.0**	**-0.00095**	± **0.00052**
Average distance to all containers (m)	10.1	-0.000040	± 0.00027			
Explained deviance (%)		0			18.66	
Error structure		Negative binomial			Quasi-Poisson	

The results of species-specific generalized linear modelling by Bayesian model averaging (BMA). Modelling was performed separately between mosquito abundance and latitude and longitude, and between the former and the remaining variables here. ”Pr” is the probability that the coefficient for a given predictor is not zero (out of 100). “EV” and “SD” are the BMA posterior distribution mean and standard deviation respectively for each coefficient, with empty cells indicating that term was removed from the model due to high colinearity. In bold: Pr, EV and SD of strong predictors (with a probability higher than 90 out of 100) and moderate predictors (also included in the optimized best-supported model for a given species or species group, with a probability between 50 and 90 (out of 100) and EV ± SD not spanning zero).

Discussion

Analysis of mosquito collections within a British woodland site with endemic avian malaria revealed the occurrence of non-random spatial patterns of mosquito abundance at a local scale. These patterns differed between species and were altogether absent for some. Distances to breeding sites and microclimatic variables were associated with mosquito abundance, with predictor sets varying between species or species groups.

Average temperature (AT) and humidity (AH) were moderate positive predictors of *Oc. annulipes* abundance, with AH also a moderate positive predictor of *An. plumbeus* abundance. This suggests that microclimate conditions of higher temperature and humidity may have favored mosquito development and survival (Clements, 1992), and therefore their abundance, near the sampling points for these species. These conditions may also favor plant development (increasing resting sites for mosquitoes) or be more amenable to hosts, increasing mosquito abundance. Thus, our results suggest that microclimate conditions are capable of exerting a significant influence in mosquito abundance even at the fine-scale of this study.

Linear distances to breeding sites were strong or moderate negative predictors of mosquito abundance. Such relationships have been previously reported (e.g. Cano *et al*., 2006; Barker *et al*., 2009) and interpreted as reflecting breeding sites as focal points of adult mosquito dispersal, both of newly emerged individuals and ovipositing females. Distance to the River Thames (DRT) was a strong predictor of *Oc. annulipes* abundance. Immature specimens were collected in a large but temporary pool (therefore not classed as pond) with abundant vegetation in the immediate vicinity of the Thames, but not on the river beds or

low current branches; this is in accordance with the known breeding biology of this species, and no specimens were expected to be found in water bodies connected to the river (Cranston *et al*., 1987; Becker *et al*., 2010). A possible higher availability of their preferred mammalian hosts near to the river may also be occurring. Taken together, these provide a possible explanation for DRT being a strong predictor of *Oc. annulipes* abundance at the study site. Average distance to all ponds (ADP) was a strong and moderate predictor for the abundance of *Oc. annulipes* and *Cs. annulata*, respectively. These habitats are within the breeding range of both *Oc. annulipes* (Schaffner *et al*., 2001) and *Cs. annulata* (Cranston *et al*., 1987), with immatures of *Cs. annulata* being found in these water bodies in the study area. Therefore, assuming these breeding sites may be influencing mosquito abundance seems justified, particularly for *Cs. annulata*.

Few environmental variables were shown to be strong predictors of mosquito abundance at the study site, with most described predictors in this analysis being considered as having no more than a moderate influence. Furthermore, only the best-supported model for *Oc. annulipes* had a high explanatory power (>90%), with the remaining best-supported final models obtained for other species or species groups having a low explanatory power (< 20%). This suggests that mosquito abundance is being driven by a complex set of predictors, with only some of these taken into account in this analysis. Unaccounted predictors may include host abundance, which may exhibit spatial patterns within and near the study site likely to influence mosquito distribution. Distances to temporary, rain-filled, water bodies (unmapped for this study) are also likely predictors, with *Cx. pipiens/torrentium*, *Cs. annulata* and *Oc. annulipes* being found in these. Mapping of water-filled tree-holes would possibly inform the analysis of *An. plumbeus* abundance, as

these are the common breeding sites for these species (Cranston *et al.*, 1987; Schaffner *et al.*, 2001).

This study has documented local-scale heterogeneity in mosquito abundance in a British woodland, and shown that it can be associated with a set of environmental predictors. Furthermore, such heterogeneity (or lack thereof) is likely to vary between mosquito species, with environmental variables likely to exert different influences regarding the species concerned. Sampling in such habitats should therefore take such fine-scale heterogeneity in consideration to avoid obtaining inaccurate assessments of their mosquito fauna, while taking into account differences in environmental variables known to have a likely effect on mosquito abundance. These would ideally include not only the variables associated with mosquito abundance in this study, but also predictors described elsewhere. This study was undertaken with previous knowledge of occurring local-scale heterogeneity in avian malaria prevalence for host populations in Wytham Woods (Wood *et al.*, 2007; Knowles *et al.*, 2011; S. Lachish *et al.*, 2012). The findings here suggest that such heterogeneity could be significantly shaped by the occurring non-random patterns of mosquito abundance. For instance, *Cs. annulata* is a known vector of *P. circumflexum* (Reichenow, 1932) and showed significant within-site heterogeneity in abundance that showed correspondence to the distribution of *Plasmodium* in its avian hosts. Further investigation of mosquito-malaria parasite distribution at the study site, guided by our current findings, is merited.

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Associations between avian malaria, mosquito abundance and landscape variables within sympatric host populations at a local scale

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Abstract

Avian malaria (*Plasmodium* spp.) is a popular model system to study ecological and evolutionary interactions between hosts, parasites, vectors and environmental variables. Recently, several studies have looked into vector associations with avian malaria hosts and parasites, but the relative role of vector abundance in shaping transmission in the wild remains largely unstudied. Here, we look into the association of mosquito density per host (putative vector availability) and landscape variables with *Plasmodium* prevalence in birds, for two parasite species (*P. relictum* and *P. circumflexum*) within populations of two sympatric avian host species at a local scale. Long-term spatially stable *P. circumflexum*

infection prevalence was strongly predicted by the density per host of *Cs. annulata* (a known vector of this parasite from experimental work) and distance to the nearby river. A different predictor (distance to woodland edge) was obtained between the two sympatric avian host populations, when considering mosquito density per host. *Plasmodium* prevalence in avian hosts in the following calendar year was not associated with mosquito density per host. Overall, landscape variables remained the strongest infection predictors of malaria in this system. Our results suggest that mosquito density per host is a variable capable of influencing spatial patterns of avian *Plasmodium* prevalence, even at a local scale and within a single avian host population. Nevertheless, knowledge is also needed regarding feeding frequency and sporozoite rate to understand avian malaria transmission. Furthermore, avian host, malaria parasite and environmental variables must also be considered to understand the ecology and evolution of avian malaria.

Keywords

Avian malaria, *Plasmodium*, blue tit, *Cyanistes caeruleus*, great tit, *Parus major*, host-parasite-vector associations, landscape ecology, mosquito density per host

Introduction

Comprehending the sources of variation in vector-borne disease prevalence is essential to understand and predict disease patterns and host-parasite-vector evolution. Parasite infection may be influenced by biotic variables such as host age, sex, immunity or population density (Keymer & Anderson, 1979; Poulin, 1996; Wilson *et al.*, 2001; Palacios

et al., 2007), but also by the landscape ecology of host-parasite interactions (Omumbo *et al.*, 2005; Wood *et al.,* 2007) and by vector and parasite availability and development, which is influenced by climate (Rogers & Randolph, 2006). Knowledge regarding the relative importance of such variables in shaping parasite prevalence is central for the study of infectious disease and of increasing relevance in a world of climatic and habitat changes, but such knowledge remains limited for wild host populations.

Vector-borne disease requires the presence of infective vectors for transmission. Vector distribution maps have been used to predict landscape-level disease prevalence (Ali *et al.*, 2003; Kulkarni *et al.,* 2010), and an effort is being undertaken to map the distribution of human malaria (*Plasmodium* spp.) vectors worldwide, a crucial tool for disease control (Hay *et al.,* 2010). Positive spatial associations between human malaria prevalence and vector abundance have been reported (Trape *et al*., 1992; Ghebreyesus *et al.,* 1999; Clarke *et al.*, 2002; Galardo *et al.*, 2009), but also the absence of such a relationship (Thomson *et al.*, 1994; Diuk-Wasser *et al.,* 2005). The entomological inoculation rate (EIR) is a commonly used metric that provides the number of infective mosquito bites per host per time unit (MacDonald, 1957). The EIR is usually used as a measure of transmission intensity in malaria and is positively associated with *Plasmodium* prevalence in human malaria, although this relationship is complex (Beier *et al.,* 1999; Smith *et al.,* 2005). Mosquito abundance is not used directly to calculate the EIR, which is a function of mosquito feeding frequency, proportion of mosquitoes with malaria parasites in their salivary glands (sporozoite rate) and mosquito density per host (Hay *et al.*, 2000; Kelly-Hope & McKenzie, 2009). EIR has a positive association with malaria parasite prevalence due to increased vector-host contact, according to theory (MacDonald, 1957).

Avian malaria (*Plasmodium* spp., *sensu* Valkiūnas *et al*., 2005) is caused by vector borne parasites, transmitted by mosquitoes. These malaria parasites are globally distributed and occur in a wide variety of avian hosts (Valkiūnas, 2005; Bensch *et al.*, 2009). The development of molecular screening techniques for these parasites resulted in their increased use as model systems to study host-parasite-vector interactions in wild settings (Bensch *et al.*, 2009). This has unveiled a large variation and diversity in avian *Plasmodium* parasite prevalence, both in avian hosts and mosquitoes, although studies on mosquitoes only recently started to emerge. Assessment of such interactions has largely considered separate avian host populations and habitats, and single populations as a whole; within-population studies remain scarce (but see Bensch *et al.*, 2007; Wood *et al.*, 2007; Knowles *et al.*, 2011; S. Lachish *et al.*, 2012). Also lacking are studies addressing the role of vector availability in shaping spatial patterns of avian *Plasmodium* prevalence in the wild (but see below).

A widely cited model proposed by van Riper III *et al*. (1986) predicted intensity of avian *Plasmodium* transmission (and strong fitness costs to native birds) in Hawaiian forests by overlaying over an altitudinal gradient the distributions of avian host and mosquito abundance. Additional work on the same study system uncovered a higher proportion of *Plasmodium* infected mosquitoes, but not highest mosquito abundance, in the area with highest *Plasmodium* prevalence in birds (Woodworth *et al.*, 2005). Avian *Plasmodium* prevalence throughout the New Zealand landmass was positively associated with the known vector distribution (Tompkins & Gleason, 2006), although no association between putative vector abundance and avian *Plasmodium* prevalence was found in Portuguese reedbeds (Ventim *et al*., 2012). These pioneer studies highlight the need to consider

variation in vector distribution when assessing patterns of avian *Plasmodium* prevalence and avian host-malaria parasite co-evolutionary dynamics. They also indicate that, like human malaria, a positive association between vector abundance and avian *Plasmodium* prevalence may not necessarily occur.

Long-term studies of avian *Plasmodium* in wild bird host populations showing spatial heterogeneity in malaria parasite prevalence offer an opportunity to investigate the influence of transmission parameters in shaping such patterns. Recent studies (Wood *et al.*, 2007; Knowles *et al.*, 2011; Lachish *et al*., 2011b; S. Lachish *et al*., 2012) have unveiled pronounced spatial variation in *Plasmodium* prevalence and transmission rates, at a local scale, in two breeding sympatric woodland tit (Paridae) populations. These showed pronounced differences in the distribution of two parasite species, *P. relictum* and *P. circumflexum*, attributed to interactions between bird, landscape and malaria parasite variables. For both avian host species, *P. circumflexum* has higher prevalence and consistent interannual transmission rates near to the river running along the northern border of the study site. *P. relictum* shows no discernible area of higher prevalence or transmission rates in most years, although analysis of pooled multi-year data for blue tits reveals an overall southerly distribution at the study site (Knowles *et al.*, 2011). Recent work has unveiled a diverse mosquito fauna sympatric with these avian host populations, showing local spatial patterns of abundance differing between species and associated with landscape and microclimatic variables (Chapter 3). Mosquito species found included: a) C*s. annulata*, a known experimental vector of *P.relictum* and *P. circumflexum* (Reichenow, 1932), and b) the *Cx. pipiens*/*torrentium* group, of which *Cx. pipiens* is a widely used experimental vector of *P. relictum*, being found associated with this parasite in the wild (Valkiūnas, 2005 and

references therein; Kim & Tsuda, 2010; Ejiri *et al*., 2011b; Glaizot *et al*., 2012; Ventim *et al*., 2012; Chapter 2). To our knowledge, the remainder of mosquito species or species groups found (*An. claviger*, *An. maculipennis* complex, *An. plumbeus, Cq. richiardii, Da. geniculata, Oc. annulipes, Oc. cantans and Oc. rusticus)* have no established association with these two *Plasmodium* species. Given the low infection rates usually found in the wild (e.g. Gager *et al.,* 2008; Glaizot *et al.,* 2012; Chapter 2) and the scarce work done on this subject (Valkiūnas, 2005), the possibility that these other species may act as vectors cannot be discarded. Furthermore, occurrence of positive associations between mosquito density per host and malaria parasite prevalence highlights a possible vector role for a given species. Mosquito abundance at the study site is highest in June-August (Chapter 5), in accordance with the known seasonal patterns of abundance in British mosquitoes (Cranston *et al.,* 1987). Therefore, *Plasmodium* transmission is likely to occur after the avian hosts breeding season, rendering its associations with malaria parasite prevalence patterns in the following year of particular interest.

Here, we look for associations between mosquito density per host (putative vector availability) and landscape variables, with *Plasmodium* prevalence within these two sympatric avian host populations, at a local scale. We aimed to (i) assess the relative importance of the above variables in predicting (a) long-term spatially stable patterns of *Plasmodium* prevalence, (b) *Plasmodium* prevalence when considering the previous years mosquito abundance and (ii) assess the relative importance of these variables for two sympatric avian host species.

Methods

Study site, avian host species and Plasmodium detection

Wytham Woods is a mixed deciduous woodland (385ha) near Oxford, United Kingdom (51º46'N, 1º20'W), where between 250 and 450 pairs each of blue tits (*Cyanistes caeruleus*) and great tits (*Parus major*) breed annually (Perrins, 1979), with breeding pairs occupying nestboxes throughout the wood. Individually marked adult breeding blue tits and great tits were blood sampled from 2005 to 2010 and 2008-2009, respectively, on one occasion between day 6 and 15 of their nestling period, which typically occurs between mid-May and early June. Both tit populations are single brooded with synchronous breeding. Therefore, blood samples were obtained at a standardized point in their annual cycle, with little variation in calendar date of sampling between years (10th-90th percentile sampling date range: 17 days). Blood samples were screened for infection with *Plasmodium relictum and P. circumflexum,* which comprise 98.2% of *Plasmodium* infections in the blue tit study population (Knowles *et al*., 2011), using quantitative PCR (details provided in Knowles *et al.,* 2011; S. Lachish *et al*., 2012). Occupancy modelling analyses has shown that the quantitative PCR methodology used is highly sensitive and has a very low probability of false negative diagnoses (Lachish *et al*., 2012), whilst false positive diagnoses are unlikely due to strict laboratorial procedures (Knowles *et al.*, 2011).

Mosquito data

In the UK, adult mosquito abundance is generally much lower or absent for most species from October to April. Seasonal patterns of abundance are species-specific (Cranston *et al.*,

1987 and references therein; Medlock *et al.*, 2005 and references therein), making a sampling strategy that encompasses different seasonal periods more likely to uncover the spatial distribution of a larger number of species. Mosquito collections were therefore made in three different sampling periods, between 1-5th June, 25-29th July and 9-12th September in a single year (2009). Miniature blacklight (UV) traps Model 1212 (John W. Hock ©, Gainesville, United States of America), baited with CO_2, were used for the collections, being placed at the sampling points for a 15-20 hour period encompassing at least 2 hours before and after sunset and sunrise. A constrained randomized design was used to allow for the selection of sampling points throughout the study site, to encompass the known within-wood differences in *Plasmodium* prevalence (see Introduction). From 61 locations spaced regularly in a 250m grid throughout Wytham Woods, four sampling points were randomly selected for sampling in each of nine approximately equally-sized different geographical sub-areas of the wood (see Chapter 3, Figure 3.1, for a descriptive figure). The same 36 points were used in all the 3 sampling periods (hence 108 samples). Due to logistical constraints (the number of traps available, which was 12), sampling was only undertaken in 3 geographical sub-areas daily, thus requiring 3 collection nights to sample all 9 sub-areas. A randomized (without reposition) design was used to minimize spatial clustering of the sub-areas on a given collection night, with the study site being divided into three major geographic areas (see Chapter 3, Figure 3.1) and one sub-area within each being selected daily. Collections within each sampling period were carried out on days of expected low wind speed (<15 km/h) and no rainfall. Collected mosquito specimens were morphologically identified following Cranston *et al.* (1987) or classed as unidentified (due to loss of diagnostic features). Morphological differentiation between *Cx. pipiens* and *Cx.*

torrentium cannot be made with certainty (Becker *et al.,* 2010); therefore, specimens identified as either of these (Cranston *et al.,* 1987) were considered as a *Cx. pipiens/torrentium* species group. In accordance with the known variability in seasonal abundance patterns of the British mosquito fauna, collections obtained for most species or species groups were very low for given sampling periods. Temporally pooled mosquito collections were therefore considered as allowing for a more robust and informative measure of abundance at the study site. Hence, for each species or species group, mosquito abundance at each sampling point (n=36) was obtained by the sum of adults collected over the three sampling periods.

Plasmodium infection prevalence, landscape and mosquito density per host data

Breeding tits are territorial and forage in the immediate vicinity of their nest (Stauss *et al.,* 2005). In Wytham Woods, tits are highly site faithful after natal dispersal (Perrins, 1979), with the home range of great tit recruits known to be broadly similar in location to their subsequent breeding territories by August (T.A. Wilkin, unpublished), and *Plasmodium* infections are seemingly acquired after individuals settle in their breeding territories in blue tits (Knowles, 2009). Hence, breeding nestbox location is likely to be quite strongly associated with individual location at time of infection. A GIS of Wytham Woods has previously been constructed (Wilkin *et al.,* 2007), including nestbox location (±3m) and oak density (Wilkin *et al.,* 2009). In order to establish separate areas in which the mosquito abundance values obtained are more likely to reflect mosquito availability, tessellations were formed around mosquito sampling points within the study site perimeter (Figure 4.1) (Stoyan *et al.,* 1987; Wilkin *et al.,* 2006). These were constructed using GIS software

(MapInfo Professional v10.0), originating a geometric construct that contains all points closer to a given sampling point than any other. These are formed on a flat surface and do not account for topographic change. Within each tessellated area, the number of blue tits and great tits infected or not with *P. circumflexum* and *P. relictum* were separately derived, henceforth referred to as infection prevalence. For multi-year infection prevalence data sets (see below), if *Plasmodium* prevalence data was available for a given individual for more than one year, only one of these was randomly kept. Different data sets were used to (i) investigate the relative importance of the variables in predicting a long-term spatially stable pattern of *Plasmodium* prevalence (2005-2010 *P. circumflexum* data set for blue tits); (ii) assess differences in the relative importance of the variables between sympatric host populations (2008-2009 *P. circumflexum* data set for blue tits and great tits); (iii) assess the relative importance of the variables in predicting patterns of *Plasmodium* prevalence, when considering mosquito abundance from the previous year (2010 *P. circumflexum* and *P. relictum* data sets for blue tits). For (i) and (ii), analysis of *P. relictum* multi-year data sets was not considered due to its temporally unstable spatial distribution.

Several landscape variables were derived, known from previous work to affect spatial variation in *Plasmodium* prevalence in tits at the study site (Wood *et al.,* 2007; S. Lachish *et al.*, 2012): (i) oak density (number of oaks/km^2), derived for the tessellated areas; (ii) distance (m) to the river Thames, (iii) altitude (m) and (iv) distance (m) to the woodland edge, derived for the mosquito sampling points, where collections are considered to reflect more strongly mosquito abundance (see below). Blue tits and great tits are known mosquito hosts, breeding throughout the study site where they display similar body size, ecology and *Plasmodium* prevalence (Perrins, 1979; S. Lachish *et al*., 2012). They are likely to provide

similar feeding oportunities to mosquitoes and were therefore considered together to derive host density (blue tits + great tits) (occupied nestbox/km^2, determined by nesting records) for the tessellated areas, with a yearly average calculated for 2005-2010 and 2008-2009. Collections with CDC light traps baited with CO_2 are considered to reflect mosquito abundance in its nearby vicinity, with a collection range smaller than 30m (Odetoyinbo, 1969; Service, 1993). Therefore, collections are not proportional to tessellated area size, but provide a measure of mosquito abundance within each area. Mosquito density per host was calculated as mosquito abundance (per sampling point) / host density (per tessellated area), with the denominator varying when considering different time intervals (2005-2010, 2008-2009 or 2010) in the statistical analysis.

Statistical analysis

Generalized linear modelling (GLM) was used to look into associations between infection prevalence, mosquito density per host and the landscape variables derived, using a quasi-binomial error distribution and a logit link. This methodology allows for the use of overdispersed proportional data in GLMs, with Gaussian, quasi-Poisson or negative binomial error distributions (see Chapters 3 and 5) being unsuitable for this type of data (Zuur *et al*., 2009). Several starting models were constructed and optimized in R 2.12.1 (http://www.R-project.org), with infection prevalence as the dependent variable (varying when considering different time intervals and avian hosts). Independent variables (N=10) were: oak density, distance to the river Thames, altitude, distance to the woodland edge, and the separate mosquito density per host of the six most abundant mosquito species or species groups (varying when considering different time intervals due to different average

host densities). Collinearity between independent variables was assessed using variance inflation factors (VIF), for both starting and optimized models. No variables presented a VIF exceeding 5, and therefore none was removed from the models. Models were then optimized by predictor selection using Bayesian model averaging (BMA), as implemented in the BMA package for R (Raftery *et al.*, 2005). This methodology averages over competing predictor combinations, accounting for the uncertainty inherent in the model selection process (Raftery, 1995). The optimization output provides the probability of a predictor coefficient not being zero. Predictors with a probability higher than 90 (out of 100) were always included in the optimized top model for a given infection prevalence data set and are mentioned here as strong predictors. Optimized best supported models also included predictors with a probability between 50 and 90 (out of 100) and with coefficient±standard deviation not spannig zero, mentioned here as moderate predictors. The following were assessed for the optimized top models: (i) percentage explained deviance, [100 x (1 – residual deviance/null deviance)], as a measure of explanatory power for mosquito abundance (analogous to r^2) (Zuur *et al*., 2009); (ii) deviance residual patterns plotted against fitted values for model validation (Zuur *et al.*, 2009); (iii) presence of residual spatial autocorrelation (RSA), through visual examination of residual binned semivariograms against a simulation envelope obtained from 999 Monte Carlo data permutations, using the geoR package (Pfeiffer *et al*., 2008) in R. No final model showed presence of RSA.

Results

From 2005 to 2010, a yearly average of 467 (standard deviation: ± 87.51) blue tits was screened for *Plasmodium*; a total of 645 and 471 great tits was screened in 2008 and 2009, respectively (Table 4.1, adapted from S. Lachish *et al*., 2012).

Table 4.1 - Avian hosts screened and prevalence for *P. circumflexum* or *P. relictum* infection in Wytham Woods, for each study year included in the analysis (adapted from S. Lachish *et al.*, 2012).

Plasmodium	**Avian host**	**Year**	**# Infected**	**Total**	**Prevalence**
P. circumflexum	Blue Tit	2005	123	467	0.263
		2006	102	471	0.217
		2007	92	522	0.176
		2008	114	517	0.221
		2009	137	529	0.259
		2010	52	297	0.175
	Great Tit	2008	214	645	0.332
		2009	167	471	0.355
P. relictum	Blue Tit	2010	34	297	0.114

A total of 836 adult mosquitoes were collected during standardized trapping, with the six most abundant species or species groups identified being *Oc. annulipes, An plumbeus*, *Cx. pipiens/torrentium*, *Cs. annulata, An. claviger* and *Da. geniculata,* which together comprised 91.4% of total collections, and 94.2% of identifiable specimens (Table 4.2, see Chapter 3 for an analysis of this data looking into associations with microclimatic and landscape variables). GLM analysis was pursued including these species or species group only.

Table 4.2 - Total number of adult mosquitoes collected ("N") and percentage ("%") of total catch for each species or species group.

Species	N	%
An. claviger	45	5.4
An. maculipennis complex	1	<1
An. plumbeus	195	23.3
Cq. richiardii	9	1.1
Cs. annulata	76	9.1
Cx. pipiens/torrentium	120	14.4
Da. geniculata	38	4.5
Oc. annulipes	290	34.7
Oc. cantans	17	2.0
Oc. rusticus	20	2.4
Unidentifiable	25	3.0
Total	836	

GLM revealed several associations between infection prevalence, mosquito density per host and landscape variables. Long-term (2005-2010) *P. circumflexum* infection prevalence (blue tits) had *Cs. annulata* density per host as a strong predictor (Figure 4.1), with no other mosquito variables being predictors (Figure 4.2 shows the spatial variation between tessellations of these malaria parasite and mosquito variables), with distance to river Thames (negative association) also a strong predictor; oak density was also included in the optimized top model, but as a moderate predictor (Table 4.3). Different sets of predictors were obtained for *P. circumflexum* prevalence (2008-2009) in blue tits and great tits (Table 4.3). For blue tits, distance to the river Thames (negative association) was a strong predictor. For great tits, distance to the river Thames (negative association) was also a strong predictor (Figure 4.1) and distance to the woodland edge a moderate predictor. For

P. circumflexum prevalence in 2010 (blue tits), distance to the river Thames (negative association) was a strong predictor and altitude a moderate predictor, with no mosquito species or species group density per host retained in the top optimized model (Table 4.3). There were no significant predictors for *P. relictum* infection prevalence.

Figure 4.1 – Relationship between (a) *Cs. annulata* density per host and long-term *P. circumflexum* prevalence (2005-2010), and (b) distance to the river Thames (m) and great tit *P. circumflexum* prevalence (2008-2009) at the study site.

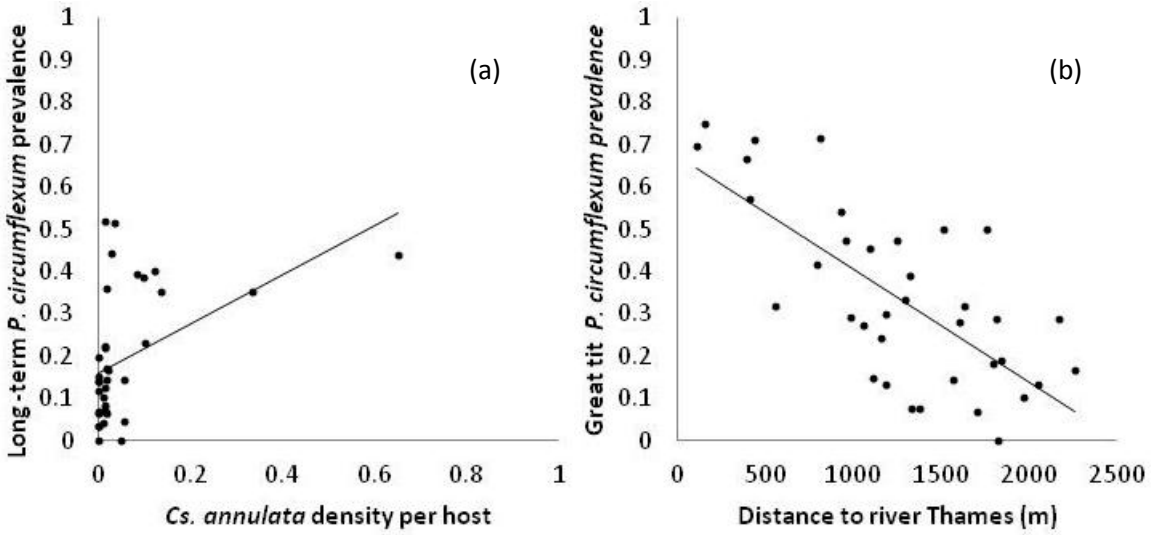

Figure 4.2 - Wytham Woods (Oxford, United Kingdom) maps showing (a) long-term *P. circumflexum* prevalence and (b) mosquito density per host for the six most abundant species, within the tessellations constructed around mosquito sampling points within the study site perimeter, using GIS software. Tessellations are formed on a flat surface and do not account for topographic change. Different shading categories correspond to upper, middle and lower third of values for prevalence and density per host.

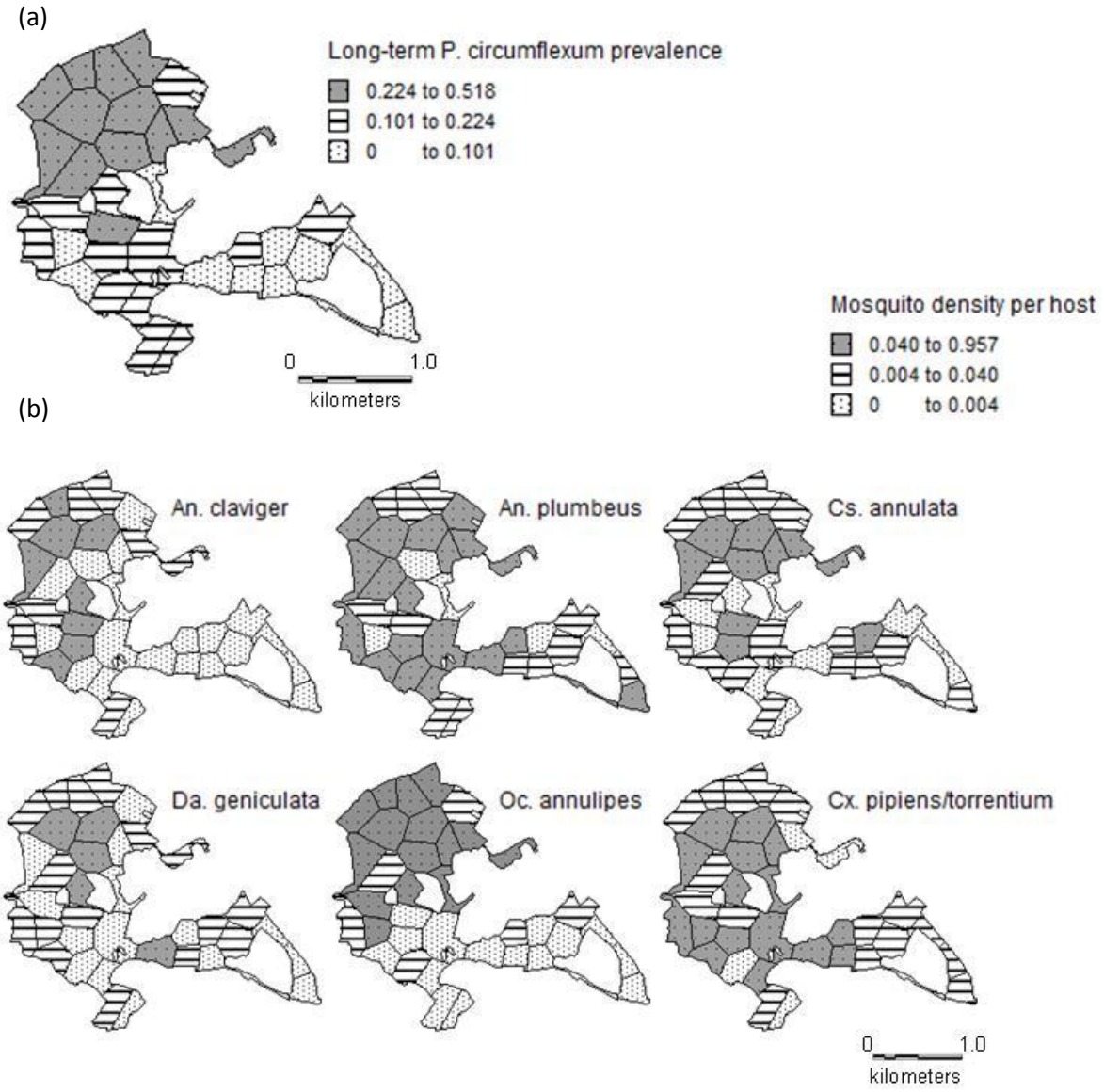

Table 4.3 - Results of model optimization using Bayesian model averaging (BMA) examining the influence of mosquito density per host and landscape variables on *Plasmodium* prevalence data sets at Wytham Woods. "River" = distance (m) to the River Thames, "edge" = distance (m) to the woodland edge, "oak" = density of oak trees (oak/km^2). "Pr" is the probability (out of 100) that the coefficient for a given predictor is not zero. "EV" and "SD" are the BMA posterior distribution mean and standard deviation, respectively, for each coefficient. Values for strong and moderate predictors included in the final optimized top model for each prevalence data set are in bold.

Avian hosts	**Blue tits - 2005/2010**		**Blue tits - 2008/2009**		**Great tits - 2008/2009**	
Plasmodium	***P. circumflexum***		***P. circumflexum***		***P. circumflexum***	
Predictor	**Pr**	**EV ± SD**	**Pr**	**EV ± SD**	**Pr**	**EV ± SD**
River	**100.0**	**-0.0012 ± 0.00019**	**100.0**	**-0.00094 ± 0.00040**	**100.0**	**-0.0010 ± 0.00030**
Cs. annulata	**100.0**	**16.77 ± 4.91**	16.1	1.54 ± 4.56	11.2	0.77 ± 2.86
Oak	**71.8**	**0.00018 ± 0.00014**	11.0	0.000030 ± 0.00012	9.1	0.000012 ±0.000057
Oc. annulipes	47.7	0.81 ± 1.11	6.5	-0.21 ± 0.81	41.8	1.39 ±2.01
Cx pipiens/torrentium	13.9	-0.85 ± 2.70	48.3	10.13 ± 12.55	5.2	0.15 ± 1.54
An. plumbeus	13.5	-0.39 ± 1.30	10.4	-0.73 ± 3.11	5.4	-0.098 ± 0.86
An. claviger	10.8	0.69 ± 2.83	9.0	1.13 ± 5.24	36.5	6.35 ± 10.18
Edge	7.9	0.000039 ± 0.00024	5.0	-0.00003 ± 0.00012	**85.6**	**0.0034 ± 0.0014**
Altitude	6.7	0.00012 ± 0.00093	5.8	0.00029 ± 0.0020	7.9	-0.00030 ± 0.0017
Da. geniculata	6.2	0.26 ± 2.51	4.8	0.012 ±4.49	20.9	3.38 ± 8.17
Explained deviance (%):		84.20		32.18		69.00

Avian hosts	**Blue tits - 2010**		**Blue tits - 2010**	
Plasmodium	***P. circumflexum***		***P. relictum***	
Predictor	**Pr**	**EV ± SD**	**Pr**	**EV ± SD**
River	**96.3**	**-0.0017 ± 0.00060**	31.3	0.00021 ± 0.00038
Cs. annulata	5.1	-1.78 ± 3.32	6.2	-0.63 ± 5.06
Oak	6.1	0.000012 ±0.000089	9.9	-0.000024 ± 0.00010
Oc. annulipes	31.9	2.23 ± 4.08	9.2	-0.38 ±1.96
Cx pipiens/torrentium	6.3	0.48 ± 3.31	5.8	-0.38 ± 3.32
An. plumbeus	6.2	0.26 ±1.82	6.6	-0.34 ±2.27
An. claviger	5.9	0.66 ± 5.06	24.7	-10.77 ±24.61
Edge	12.2	0.00025 ±0.0011	6.9	0.000067 ±0.00066
Altitude	**83.2**	**0.016 ±0.010**	18.0	0.0020 ±0.0053
Da. geniculata	5.4	-0.46 ± 4.83	5.6	-0.54 ±5.76
Explained deviance (%):		47.64		0

Discussion

Analysis of *Plasmodium* infection prevalence at a local scale among wild birds revealed mosquito density per host of several species or species groups and landscape variables as strong or moderate predictors, with their relative importance varying between avian host species and infection prevalence data sets.

The long-term (and arguably more robust) 2005-2010 data set on *P. circumflexum* infection prevalence in blue tits showed the highest number of associated predictors, with *Cs. annulata* density per host and distance to the river Thames as strong predictors. Distance to the river Thames has been previously reported as the strongest predictor for *P. circumflexum* data sets at the study site (Wood *et al.*, 2007; S. Lachish *et al*., 2012), with transmission rates for this parasite estimated, from multi-state models, to be higher near the river (Lachish *et al.*, 2011). These associations were hypothesized to be the result of more favorable conditions for vector proliferation due to higher availability of wet larval habitats linked with the presence of this water body. Increased human malaria risk has been reported near water bodies, supporting this hypothesis (e.g. van der Hoek *et al*., 2003; Omumbo *et al*., 2005; Midega *et al*., 2012). Here, GLM revealed *Cs. annulata* density per host as an infection prevalence predictor for a long-term *P. circumflexum* data set, thus showing a link between putative vector density per host and *P. circumflexum* prevalence; this mosquito species is more abundant on the north of the study site (Chapter 3). *Cs annulata* is a proven experimental vector of *P. circumflexum* (Reichenow, 1932) and also an ornithophagic mosquito in the British Isles (Medlock *et al.*, 2005). A role for *Cs annulata* in *P. circumflexum* transmission at the study site is therefore a likely scenario as

suggested by being a strong infection prevalence predictor here, with its density per host likely to exert an influence in shaping the spatial prevalence patterns observed. Oak density was a moderate predictor for the 2005-2010 *P. circumflexum* infection prevalence in blue tits. Oak density may reflect tree density at the study site, which may result in more mosquito breeding sites in a given area through water-filled tree-holes. Several species in Great Britain are known to use these as breeding habitats (Cranston *et al.,* 1987), therefore likely increasing local availability of vectors.

Distance to the river Thames was the only predictor retained (a negative association of strong significance) across the analyses of long-term (2005-2010) and the 2008-2009 and 2010 *P. circumflexum* infection prevalence data sets for blue tits, with several differences in predictor sets occurring. *P. circumflexum* has largely stable, but not wholly overlapping, spatial patterns of prevalence and infection risk clusters at the study site between years (Knowles *et al.,* 2011; S. Lachish *et al.,* 2012). Therefore, limited but significant differences in ecological processes occurring between years may have led to differences in infection prevalence predictors, but the different robustness of the 2008-2009 and 2010 data sets may have also contributed to the results. The temporally comparable 2008-2009 *P. circumflexum* infection prevalence data set for great tits had distance to the river Thames (negative association) as a strong predictor and distance to the edge of the wood as a moderate predictor. Distance to the river Thames was previously found to be the strongest predictor of infection risk for both blue tits and great tits at the study site (S. Lachish *et al.*, 2012), in accordance with the results here despite our inclusion of mosquito density variables in the analysis. Distance to the edge of the wood is a moderate predictor for great tits, possibly due to higher rate of encounters (non-density dependent) with vectors further

from the edge of the wood. Mosquitoes may obtain more avian blood meals and shelter here due to the occurrence of lower wind speeds, required for active dispersal and host-seeking flight (Service, 1997). While these are a possible explanation for the edge effect found, they do not explain differences between avian hosts. These may be due to differences in host settlement patterns, a possibility that requires further investigation. Overall, results here highlight the need to consider possible differences in avian *Plasmodium* infection prevalence predictors between avian hosts at a local scale, even between closely related bird species.

The 2010 *Plasmodium circumflexum* infection prevalence data sets (blue tits) had altitude as a moderate predictor. Altitudinal differences in soil type and temperature occur at study site (R. Alves, unpublished) and may influence epidemiological processes. Such influence may have been stronger for 2010, or alternatively, blue tit settlement patterns may have differed from previous years, rendering this altitudinal effect detectable. Results for the 2010 data sets further suggest that mosquito density per host is not the main variable influencing *P. circumflexum* prevalence in blue tits at the study site. No predictors were obtained for P. *relictum*, in accordance with the known apparently random spatiotemporal infection risk for this parasite at the study site (S. Lachish *et al.*, 2012). *Cs. annulata* and the *Cx. pipiens/torrentium* group are known experimental vectors of this malaria parasite. The positive association of *Cs. annulata* with *P. circumflexum* at the study site does not suggest a role of this mosquito in shaping *P. relictum* prevalence, as the *Plasmodium* species have a largely allopatric distribution in Wytham Woods. The *Cx. pipiens/torrentium* group may be composed at the study site largely of *Cx. torrentium*, an untested species as *Plasmodium* vector. A hypothesis for the random *P. relictum* distribution is the influence of

variable mosquito abundance shaping prevalence between years (Knowles *et al.*, 2011; Lachish *et al.*, 2011b), but we found no evidence here of such association for the 2010 *P. relictum* data. The absence of areas of elevated infection risk for *P. relictum* in 2010 (S. Lachish *et al*., 2012) suggested *a priori* that mosquito density per host variation within-wood would not be strongly associated with infection prevalence for this malaria parasite, and the results here do not disprove this assumption.

Distance to the river Thames was retained in all the *P. circumflexum* models considered here and was the strongest predictor for both blue tits and great tits despite the inclusion of mosquito density per host variables in the analysis. Mosquito density per host was strongly associated with infection prevalence only when looking into a long-term data set (2005-2010). This suggests proximity to the river may also influence *P. circumflexum* prevalence by ecological processes other than by increased vector/host ratio (Wood *et al.*, 2007; Knowles *et al.*, 2011; S. Lachish *et al*., 2012). Knowledge regarding feeding frequency and sporozoite rate of the specimens collected, and how these relate with avian host, malaria parasite and environmental variables, would inform our understanding of vector role in this avian malaria system. Screening of a different collection of mosquito specimens from the study site, also with CDC light traps baited with CO_2, yielded very low infection rates (with *Plasmodium* sp., MLE 0.42%, Chapter 2), with only *Cx. pipiens/torrentium* found infected with *Plasmodium* sp.. This suggests the possibility of discriminate and ineffective vector sampling by the methodology used, as CDC light traps baited with CO_2 may not to attract equally the mosquito fauna at a given location (Service, 1993). *Cs. morsitans*, a known experimental vector of *P. circumflexum* (Meyer & Bennett, 1976), has been collected sparsely at the study site by CDC light traps but more frequently by other sampling

methods (R. Alves, unpublished). Spatial variation in this species density (if any) could hypothetically be associated with *P. circumflexum* prevalence and reduce the predictive power of distance to the river Thames. Furthermore, our mosquito abundance data is from a single year only. Multi-year collections with the methodology used would allow for stronger data robustness and insights into local scale variation at the study site, while allowing for an assessment of its long-term stability.

The density per host of *Cs. annulata* was a strong predictor of long-term *P. circumflexum* infection prevalence at a local scale on the study site. If the spatial pattern of the former is largely temporally stable, as the pattern for *P. circumflexum* in birds is likely to be (S. Lachish *et al*., 2012), then it may be exerting an important influence on the local scale variation in infection prevalence observed. While temporal changes in this avian malaria system occur over time (Wood *et al*., 2007; Knowles *et al*., 2011, Lachish *et al*., 2011b; S. Lachish *et al*., 2012), the continuous existence of adequate conditions (such as proximity to a large water body and woodland presence) may temporally secure avian host, malaria parasite and mosquito population survival and association in the north of the study site, resulting in the long-term maintenance of the spatial infection prevalence patterns observed. If these are maintained for longer evolutionally time scales, they could hypothetically lead to spatially dependent parasite-vector co-evolution, even at the local scale of our study site.

Conclusion

Our results suggest mosquito density per host can influence spatial patterns of avian *Plasmodium* prevalence, even at a local scale and within a single bird population.

Nevertheless, mosquito density per host was not the main infection prevalence predictor here in any of our analyses and therefore cannot explain avian *Plasmodium* prevalence single-handedly, highlighting the need to also assess the other components of the entomological inoculation rate (feeding frequency and sporozoite rate) to understand transmission of avian malaria. These must be considered alongside avian host, malaria parasite and environmental variables to understand the prevalence and evolution of this avian disease.

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Seasonal abundance of woodland British mosquitoes, associations with environmental variables and implications for avian *Plasmodium* transmission

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Abstract

Mosquito borne disease shows seasonal patterns of transmission due to the impact of environmental variables on their vectors. In temperate climates, mosquitoes typically present seasonal patterns of adult abundance regulated by temperature and rainfall and by diapause in the colder months. Here, we assessed seasonal patterns of mosquito abundance

and their associations with temperature and rainfall as well as calendar date, in a British woodland site where avian malaria is endemic. Mosquito abundance displayed seasonal patterns and was highest between June-August, consistent with expected patterns for temperate climates and previously reported patterns in Great Britain. *Oc. annulipes*, *An. claviger, An. plumbeus, Cx. pipiens*/*torrentium, Da. geniculata* and *Cs. annulata* comprised 84.8% of total collections, with only *Cs. annulata* being collected between October-April (<1% of total catches). Average temperature and total rainfall 30 days prior to sampling date were positively associated with *Oc. annulipes* and *An. plumbeus* abundance, respectively, alongside calendar date. Avian malaria (*Plasmodium* spp.) prevalence at the woodland site was assessed for breeding blue tits at a standardized point in their annual cycle (between early May and early June), with a *P. circumflexum* prevalence of 49.1% obtained prior to the seasonal increase in adult mosquito abundance. Findings here provide evidence that avian malaria transmission in British woodlands is limited mainly to June-August and follows the occurrence of a spring relapse of previously acquired infections or, alternatively, the maintenance of chronic blood parasitaemia through the colder months, in agreement with theoretical expectations and results elsewhere for temperate climates.

Keywords

Mosquito abundance, seasonal pattern, environmental variables, woodland, avian malaria

Introduction

Vector-borne disease is regarded as particularly susceptible to seasonality due to the impact of environmental conditions on its vectors (Rogers & Randolph, 2006). Mosquitoes (Diptera: Culicidae) vector a wide range of human and veterinary disease such as malaria, West Nile fever and dirofilariasis. Temperature and rainfall are regarded as the main environmental variables influencing mosquito development, survival and activity (Hoshen & Morse, 2004) and have been associated with seasonal mosquito abundance, fecundity and biting rate (e.g. van der Hurck *et al.*, 2000; Mbogo *et al.*, 2003; Bolling *et al.*, 2005; Reisen *et al.*, 2008; Battaile *et al.*, 2010; Lysyk, 2010). The strength of these climatic associations varies, with multi-species studies often reporting them as variously positive, negative or absent. Rainfall may affect breeding site availability and suitability for larval development, influencing population size (Hoshen & Morse, 2004; Juliano, 2009). Mosquito survival and development occurs between given temperature limits, and their development rate and feeding frequency increase with higher temperatures until an optimum level is reached (Service, 1980). In temperate climates, temperatures in the colder months cause many mosquitoes to enter diapause, a condition of suppressed development that usually precedes the advent of adverse conditions and may not terminate with the end of adversity (Koštál, 2006). This process is regulated by changes in day-length, in addition to changes in temperature and moisture (Danks, 1987; Denlinger, 2002). The majority of British mosquitoes present lower abundance or no adult activity during the colder months (October-March), with differences in the presence of diapause and the means by which it is regulated contributing to differences in seasonal abundance patterns among species

(Cranston *et al.*, 1987), leading for example to differences in voltinism (species are univoltine, with a single generation per year, bivoltine or multivoltine) (Marshal, 1938; Service, 1969; Service, 1977; Yates, 1979; Sulaiman & Service, 1983; Ramsdale & Wilkes, 1985; Cranston *et al.*, 1987; Onyeka & Boreham, 1987; Service, 1994; Hutchison *et al.*, 2007; Snow & Medlock, 2008; Clarkson & Setzkorn, 2011). Diapause in most British mosquitoes is therefore expected to render calendar date a significant abundance predictor, while temperature and rainfall are also likely to be influential, with these associations (or lack thereof) likely to vary between species. Seasonal predictors of abundance for British mosquitoes are yet to be formally assessed in wild conditions, with such knowledge aiding in understanding seasonal patterns of mosquito abundance and therefore informing the study of mosquito borne disease, a matter of particular importance in a world of changing climate.

Avian malaria (*Plasmodium* spp., *sensu* Valkiūnas *et al.*, 2005) can present seasonally stable prevalence rates in tropical regions (Fallon *et al.*, 2004). In temperate regions, transmission is regarded as occurring mostly in the warmer seasons, as this is a more favorable time for mosquito development and activity (Beaudoin *et al.*, 1971; Valkiūnas, 2005). A prevalence peak in the spring is often reported and is regarded as a relapse of previous infections due to *Plasmodium* re-emergence in the blood, as vector abundances are low in the spring (Applegate, 1971; Beaudoin *et al.*, 1971; Cosgrove *et al.*, 2008). Avian malaria vectors remain scarcely studied in the wild, including the influence of their ecology in shaping seasonal prevalence patterns. In a pioneer study, Janovy (1966) contrasted patterns of seasonal mosquito abundance and avian malaria prevalence in a prairie marsh habitat, concluding that a spring prevalence peak precedes at least the majority of

transmission, according to theoretical expectations (Beaudoin *et al.*, 1971). Such work allows for insights into timing of transmission, informing the study of seasonal dynamics between avian hosts, malaria parasites and vectors, but has not been replicated in a different temperate habitat.

Here, mosquitoes and avian hosts were sampled in a known avian malaria endemic woodland site in order to study seasonal patterns of mosquito abundance and their relation to *Plasmodium* prevalence. Marked seasonal variation is known to occur for *Plasmodium* prevalence at the study site, with *Plasmodium circumflexum* presenting both spring and autumn prevalence peaks (only in first year birds in the autumn), while *P. relictum* has a more stable seasonal distribution (Cosgrove *et al.*, 2008). Our aims were to: (i) obtain further insights into the seasonal abundance patterns of woodland British mosquitoes, and assess their associations with temperature and rainfall alongside calendar date; (ii) contrast seasonal patterns of mosquito abundance (putative vectors) with *Plasmodium* prevalence in breeding birds, to obtain insights into timing of transmission in British woodlands.

Methods

Study area

Sampling was conducted in Wytham Woods (51°46′N, 1°20′W, Oxford, United Kingdom), a mixed deciduous woodland surrounded mainly by agricultural and pasture land. This woodland and its surroundings provide a wide availability of blood meal sources (e.g. deer, cattle, blue tits, ducks), resting and breeding sites for mosquitoes (Savill *et al.*, 2010). Between 250 and 450 pairs of blue tits breed annually here (Perrins, 1979), with breeding pairs occupying nestboxes throughout the wood typically between April-early June.

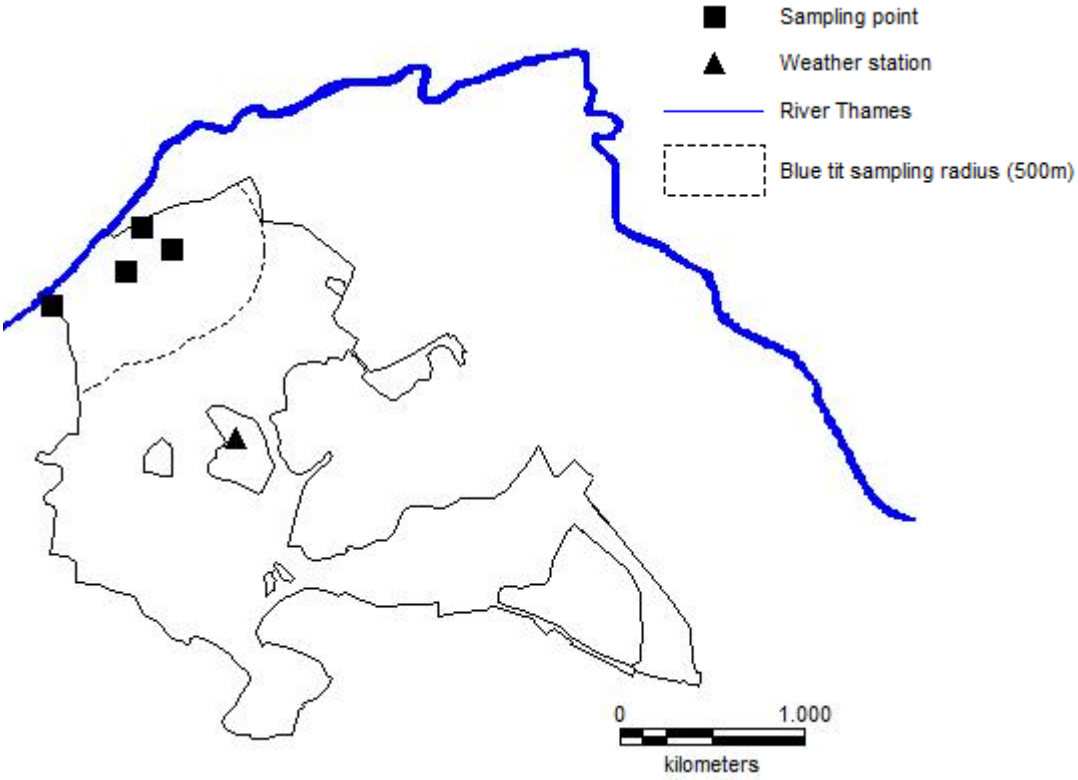
Outside the breeding season, blue tits are also commonly found in the study site (Cosgrove *et al.*, 2008). Sampling of adult mosquitoes and avian hosts was conducted in the northwestern area near the Thames. The area is known to present the highest overall mosquito abundance (M.J. Wood, D.A. Cullen, R.M. Mallis, B.C. Sheldon, unpublished) and *Plasmodium* prevalence in breeding blue tits (Wood *et al.*, 2007) across the wood, and thus was best suited to our study aims.

Mosquito collections and environmental data

From 2008 to 2010, periodic mosquito sampling was made approximately fortnightly from May-September (N=27) in the study area. Additional mosquito sampling during the study period was made in October (N=2), January (N=1), March (N=1) and April (N=3). In order to assess repeatability of collection data, extra mosquito sampling was made two days apart from periodic samples in mid-July and early August 2008. In all sampling dates, collections were made in the study area in the same four sampling sites, two by the woodland edge and two further into the wood (Figure 5.1), as the mosquito species present at the site may breed, rest and seek hosts preferentially within the wood or in its surroundings. Collections of adult mosquitoes were made with CDC (Centers for Disease Control) model light traps (Sudia & Chamberlain, 1962) baited with CO_2, two being incandescent light Model 1012 (John W. Hock ©, Gainesville, United States of America), and two other incandescent light Model 512 (John W. Hock ©, Gainesville, United States of America), one fitted with a UV LED (4 V, 420nm wavelength peak; Maplin©, Manvers, United Kingdom). On each sampling date, each trap was randomly assigned to each sampling point, allowing for the assessment of differences in trap type efficiency. Sampling was carried out on nights with

conditions of low expected wind speed (<15 km/h) and no rainfall, to avoid collection bias due to these meteorological variables (Service, 1993). Traps were operated for a minimum of 12 hours, always including at least one hour before sunset and after sunrise, generally encompassing the period of known highest mosquito daily activity (Service, 1980). Collected mosquito specimens were morphologically identified following Cranston *et al.* (1987) or classed as unidentified (due to loss of diagnostic features). Morphologic differentiation between *Cx. pipiens* and *Cx. torrentium* cannot be made with certainty (Becker *et al.*, 2010) and therefore specimens identified as either of these (Cranston *et al.*, 1987) were considered as a *Cx. pipiens/torrentium* species group.

Figure 5.1 - Wytham Woods (Oxford, United Kingdom) boundaries and nearby river Thames, with location of weather station, mosquito sampling points and blue tit sampling radius.



Hourly total rainfall (mm) and average air temperature (°C) were obtained from a weather station (AWS, Didcot Instruments Co., Abingdon, United Kingdom), situated 1040m from the nearest mosquito sampling point (Figure 5.1).

Bird sampling and avian Plasmodium diagnosis

Breeding blue tits were captured at nestboxes in an area within a 500m radius from any of the mosquito sampling points, inside Wytham Woods (Figure 5.1). Captures occurred in 2009 and 2010 between early May and early June, when broods were between day 6 and 15 post-hatch. Thus, all birds were blood sampled at a standardized point in their annual cycle. Blood samples were screened for infection with *Plasmodium relictum and P. circumflexum*, which comprise 98.2% of *Plasmodium* infections in the blue tit study population (Knowles *et al*., 2011), by quantitative polymerase chain reaction (protocols are provided in Knowles *et al.*, 2011).

Statistical analysis

Prior to testing for effects of environmental variables on seasonal patterns, tests were performed on data collected between May and September to assess repeatability of fortnightly collections and influence of trap type efficiency. Repeatability of collection data was assessed by comparison of number of specimens collected per fortnightly sample, for the six most abundant species or species group during the study period, between the fortnightly sample and the additional sampling efforts made two days later (N=2) in 2008 (mid-July and early August), using Pearson's product-moment correlation coefficient (r). Differences in trap efficiency were assessed using generalized linear modelling (GLM) for

each of the six most abundant species or species groups, with number of collected specimens as dependant variable and trap type as independent variable (N=54 trap nights for Model 1012; N=27 trap nights for Model 512 and Model 512 fitted with a UV LED). We expected different error structures to be appropriate between species or species groups. Therefore, each saturated model was fitted with Gaussian (using log transformed dependent variables), quasi-Poisson and negative binomial error structures, with the more appropriate for each species or species groups selected by visual examination of plotted deviance residuals against fitted values (Zuur *et al.*, 2009). Each selected model was then compared with the null model by analysis of deviance.

Environmental data variables were constructed for the 1 to 30 days and the 31 to 60 days periods before each periodic sampling date. For each period, hourly total rainfall values were summed (henceforth total rainfall) and hourly average temperature values averaged (henceforth average temperature). Cross-correlation (Venables & Ripley, 2002) between these four variables was assessed by pair-wise comparisons for the data of each sampling year (2008-2010) separately, using the ccf function in R 2.12.1 (http://www.R-project.org). Average temperature variables were highly correlated at lag 0 in each sampling year, and therefore average temperature for the 31-60 days period before sampling was dropped from further analysis, due to an *a priori* assumption it would exert less influence in seasonal mosquito abundance than average temperature for 1 to 30 days before sampling. The summed rainfall variables for the two time periods were uncorrelated with each other or with average temperature for 1 to 30 days; therefore, three environmental variables were entered into the saturated models for each species (average temperature for 1-30 days, total rainfall for 1-30 and 31-60 days). Drivers of seasonal variation in mosquito abundance were

analysed using generalized additive modelling (GAM), allowing consideration of nonlinear smoothed functions of a covariate alongside conventional linear predictors (Hastie & Tibshirani, 1990). Starting models were constructed for each of the six most abundant species or species groups (*An. claviger*, *An. plumbeus*, *Cx. pipiens/torrentium*, *Cs. annulata*, *Da. geniculata* and *Oc. annulipes*), with average number of mosquitoes collected each periodic sampling date as dependent variables. Independent variables consisted of a smoothed function of sampling calendar date (considered as julian day of the year, i.e. 1 to 366), the three retained environmental variables and year of sampling. Selection of the more appropriate error structure was conducted as described for the analysis of trap efficiency (see above). Starting models were optimized by backward stepwise elimination of non-significant predictors ($P>0.05$), which were retained if their removal caused a significant ($P<0.05$) increase in model residual deviance. Deviance residual patterns were plotted against fitted values of final optimized models for model validation. Percentage explained deviance, [100 x (1 – residual deviance/null deviance)], was calculated as a measure of explanatory power for mosquito abundance (analogous to r^2) (Zuur *et al*., 2009).

Results

A total of 1976 mosquitoes (not including extra sampling) were collected in 2008-2010 (Table 5.1), with 1974 (99.9%) collected during periodic sampling in May-September and 2 (both *Cs. annulata*) (0.01%) collected during additional sampling in October-April, this corresponds to a mean (± standard error) number of collected specimens per trap of 18.28 (± 5.58) and 0.071 (± 0.046), respectively. *Oc. annulipes*, *An. claviger*, *An. plumbeus*, *Cx.*

pipiens/torrentium, *Da. geniculata* and *Cs. annulata* comprised 84.8% of total collections and 97.2% of identifiable specimens (Table 5.1). *Oc. annulipes* was the most abundant species, comprising 64.4% of collected specimens. Samples taken two days after periodic collections in 2008 were significantly (P<0.05) correlated for the six most abundant species or species groups, both in mid-July (r=0.94; P=0.005) and early August 2008 (r=0.98; P<0.001), suggesting the sampling methodology used provides repeatable data. Absence of a significant (P<0.05) effect of trap type in the collections for each of the six most abundant species or species groups also suggests the methodology used provides repeatable data (*Cx. pipiens/torrentium*: F=0.069, d.f.=2, P=0.934; *Oc. annulipes*: F=1.52, d.f.=2, P=0.224; *Da.*

Table 5.1 - Total number of adult mosquitoes collected ("N"), with percentage ("%") of total catch for each species or species group in brackets.

	Periodic sampling (May-September)		Additional sampling (October-April)	
Species or species group	**N**	**%**	**N**	**%**
Ae. vexans	1	<1		
An. claviger	160	8.1		
An. maculipennis s.l.	2	<1		
An. plumbeus	71	3.6		
Cq. richiardii	8	<1		
Cx. pipiens/torrentium	62	3.1		
Cs. annulata	51	2.6	2	<1
Dc. geniculata	56	2.8		
Oc. annulipes	1273	64.4		
Oc. cantans	7	<1		
Oc. caspius	2	<1		
Oc. rusticus	29	1.5		
Unidentifiable	252	12.8		
Totals	1974		2	
Grand Total	1976			

geniculata: F=0.14, d.f.=2, P=0.874; *Cs. annulata*: F=0.24, d.f.=2, P=0.788; *An. plumbeus*: F=1.03, d.f.=2, P=0.360; *An. claviger*: F=0.67, d.f.=2, P=0.516; all models fitted with Gaussian distribution, considered the most appropriate by visual examination of plotted deviance residuals against fitted values).

Sampling collections for all species or species groups had their highest monthly mean between June-August (Figure 5.2). Collections were generally lower in September, with two specimens (*An. plumbeus* and *Oc. rusticus*) being collected in May. Additional sampling (October-April) collected two *Cs. annulata* specimens, in October 2010. Analysis with GAM unveiled associations between seasonal variation in mosquito abundance and calendar date and environmental variables, with differences between species (Table 5.2). Probably due to the large proportion of zero values in the time series, regardless of error structure GAM models showed substantial lack of fit for *An. claviger*, *Cx. pipiens/torrentium* and *Da. geniculata*. For *Cs. annulata*, a GAM model could be fitted successfully to seasonal patterns in abundance but no significant effects of year, calendar date or environment could be detected. For *An. plumbeus* and *Oc. annulipes*, the smoothed function of calendar date was a significant predictor of seasonal abundance but no effect of year could be detected. Temperature and rainfall 30 days prior to sampling date were retained for *Oc. annulipes* and *An. plumbeus*, respectively. The final models for *Oc. annulipes* and *An. plumbeus* had a percentage deviance explained (i.e. explanatory power) of 81.1% and 47.5%, respectively.

A total of 165 breeding blue tits were screened for avian malaria. Overall *Plasmodium* prevalence was 55.5%, with 49.1% of birds infected with *P. circumflexum* and 6.1% infected with *P. relictum*. Prevalence was not significantly different between years for *P.*

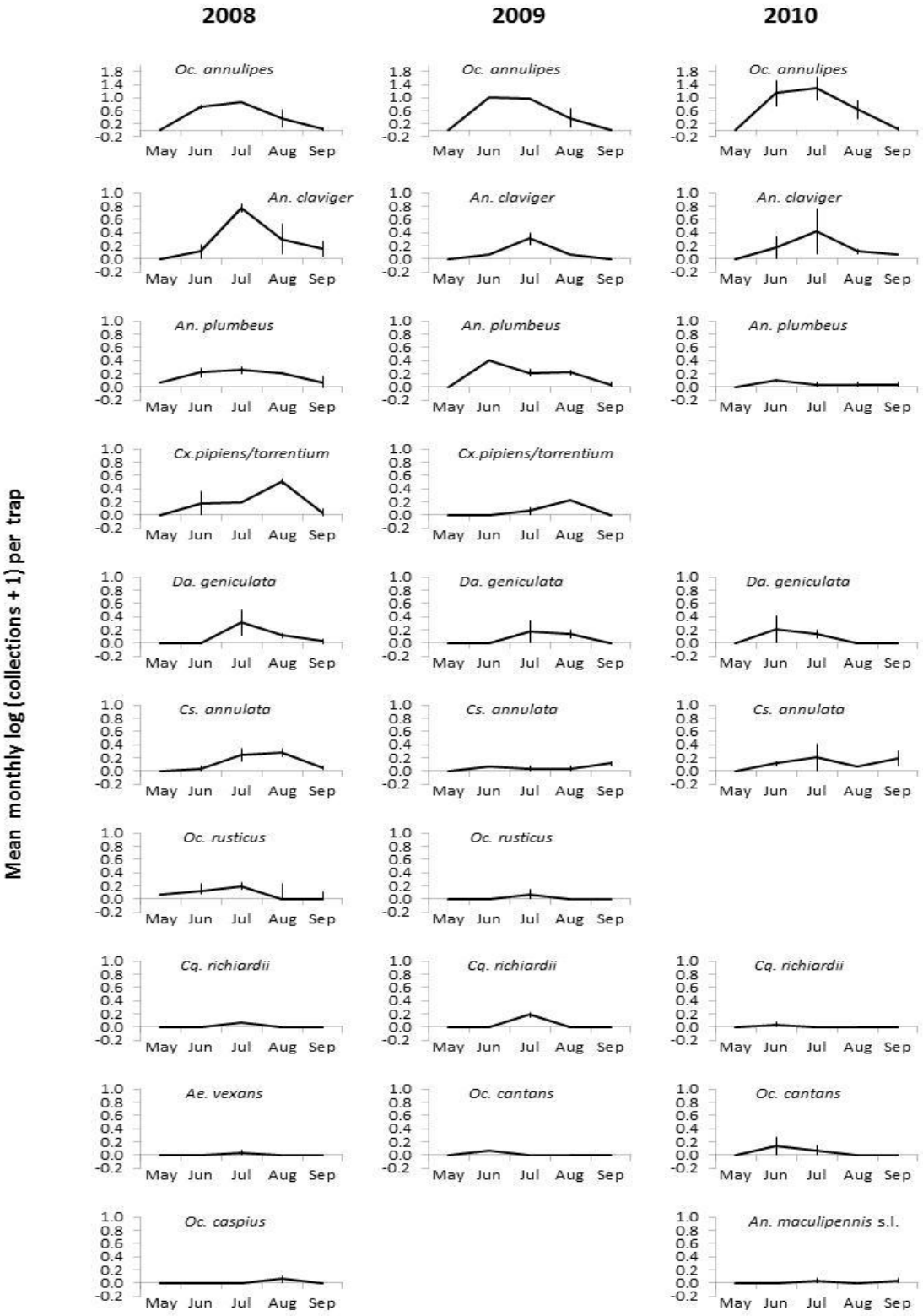
circumflexum (χ^2=0.57, d.f.=1, P=0.49) and *P. relictum* (Fisher's Exact Test, P=1).

Table 5.2 - Results from GAM (generalized additive modelling) analysis of variables predicting seasonal mosquito abundance in the study area. "Temp30" = average temperature (°C) in the thirty days period prior to mosquito sampling date. "Rain30" and "Rain60" = total rainfall (mm) in the thirty days and the 31-60 day period prior to mosquito sampling date, respectively. Retained predictors are in bold, with statistics from the final model. For non-retained predictors, statistics are from the point they left the model.

Predictor	Parameter estimate	t	P	Error structure	Deviance explained (%)
Oc. annulipes					
Temp30	**0.14 ± 0.060**	**2.24**	**0.036***	Gaussian	81.1
Rain30	0.0017 ± 0.0016	1.05	0.310		
Rain60	-0.00017 ± 0.0025	-0.07	0.947		
Year.2009	0.11 ± 0.15	0.71	0.490		
Year.2010	0.25 ± 0.20	1.25	0.228		
Smoothed sampling calendar date: estimated d.f. = 3.77, F=16.75, P < 0.001					
An. plumbeus					
Temp30	0.023 ± 0.010	2.28	0.034*	Gaussian	47.5
Rain30	**0.0018 ± 0.00048**	**3.76**	**0.001**		
Rain60	0.00070 ± 0.00080	0.87	0.392		
Year.2009	0.059 ± 0.050	1.18	0.252		
Year.2010	-0.029 ± 0.066	-0.43	0.670		
Smoothed sampling calendar date: estimated d.f. = 1.919, F=3.13 , P = 0.055					
Cs. annulata					
Temp30	0.090 ± 0.37	0.24	0.81	quasi-Poisson	0
Rain30	0.0067 ± 0.0097	0.69	0.50		
Rain60	-0.0089 ± 0.015	-0.59	0.57		
Year.2009	-0.96 ± 0.92	-1.05	0.31		
Year.2010	0.043 ± 1.21	0.04	0.97		
Smoothed sampling calendar date: estimated d.f. = 2.94, F=0.74 , P = 0.575					

* Removal of Temp30 resulted in a significant (P<0.05) increase in model residual deviance for *Oc. annulipes*, but not for *An. plumbeus*.

Figure 5.2 - Average number of mosquitoes collected, monthly per trap, in the study area for each species or species group, with periodic sampling (May-September) in 2008-2010.



Discussion

In this study we investigated seasonal patterns of British mosquito abundance in an avian malaria endemic woodland site, uncovering associations with calendar date, temperature and rainfall for two species. *P. circumflexum* and *P. relictum* prevalence was assessed for breeding blue tits in a standardized point in their annual cycle (early May-early June), allowing for insights into timing of transmission in British woodlands and the seasonal ecology of avian malaria.

Oc. annulipes was collected between June and September, with abundances having a sharp decrease in August (Figure 5.2). Such pattern has been reported previously in Great Britain (Hutchinson *et al.*, 2007; Snow & Medlock, 2008), and is likely primarily driven by *Oc. annulipes* being univoltine (Marshall, 1938), with larval development being completed by early summer (Becker *et al.*, 2010). *An. claviger* is considered bivoltine in Great Britain (Service, 1973), and a recent study has reported relatively high abundances throughout June-September, suggesting more than one generation per year (Hutchinson *et al.*, 2007). In our study, *An. claviger* had a pronounced abundance peak in July suggesting univoltinism, or alternatively a higher adult input from an earlier generation. *Da. geniculata* showed a similar abundance pattern to *An. claviger* and in accordance with a previous study (Yates, 1979), which suggested the former is largely univoltine in Great Britain.

An. plumbeus was collected from May to September, with abundances remaining relatively high throughout June-August in 2008-2009 (collections were sparse in 2010). Relatively higher abundances have been reported in both June and September for this species in Great Britain suggesting bivoltinism (Service, 1969). Our results support this assumption, as the abundance pattern observed suggests a double adult input to the population, firstly before

June and afterwards in July-August. *Cx. pipiens* is considered multivoltine in Great Britain in both its typical form *pipiens* and biotype *molestus* (Cranston *et al.*, 1987). *Cx. torrentium* remains less studied, sharing several biological parameters with *Cx. pipiens* (Cranston *et al.*, 1987; Becker *et al.*, 2010). We have found *Cx. pipiens*/*torrentium* at the study area from June-September, with a pattern suggesting bivoltinism in 2008 and univoltinism in 2009 (no specimens were collected in 2010). This contrasts with other studies in Great Britain which found either *Cx. pipiens* or *Cx. torrentium* in a wider temporal distribution (i.e. multivoltinism) and in relatively large numbers in October (Hutchinson *et al.*, 2007; Snow & Medlock, 2008; Clarkson & Setzkorn, 2011). *Cs. annulata* is considered multivoltine (Medlock *et al.*, 2005), with active adults collected in Great Britain throughout the year (Marshal, 1938; Cranston *et al.*, 1987). Here, it was found from June until October, suggesting a more limited temporal distribution. Nevertheless, it was the only species (2 mosquitoes) collected in October, while month of highest abundance differed between each year (in accordance with Service (1969)), signaling an innate capacity for multiple generations per year. Other species were collected too sparsely to allow for an assessment of their seasonal patterns of abundance.

Overall, the seasonal patterns of mosquito abundance reported here are in accordance with expected patterns for temperate climates (Altizer *et al.*, 2006), and largely agree with previous studies for the British fauna. These often report different patterns for a given species (as occurred in this study), highlighting how seasonal mosquito abundance may differ even between geographically close populations. Furthermore, such studies have also reported yearly differences in seasonal abundance patterns, as observed here. Differences in environmental conditions between populations and years may affect seasonal availability of

hosts, resting and breeding site availability. Lower sampling frequency between October-April may have contributed to our assessment of almost complete absence of adult activity during this period, in contrast with results elsewhere (Cranston *et al*., 1987 and references therein Medlock *et al.*, 2005 and references therein, Snow & Medlock, 2008; Clarkson & Setzkorn, 2011).

Calendar date was a highly significant predictor of *Oc. annulipes* abundance ($P<0.001$). The temporally limited adult input into the population for this univoltine species, which diapauses in the egg stage for most of the second half of the calendar year (Cranston *et al.*, 1987; Becker *et al.*, 2010), will likely explain this strong association. Calendar date was also retained in the final model for *An. plumbeus* (bivoltine) with a weaker significance ($P<0.1$) and was not retained for *Cs. annulata* (likely to present multiple generations in our population). Year was not retained for any of these species, suggesting absence of unknown variables varying yearly with an effect on abundance.

Average temperature 30 days prior to sampling date (Temp30) was retained for *Oc. annulipes* abundance. Higher Temp30 will not have an effect on diapause termination in this species, which occurs early in the calendar year (Cranston *et al.*, 1987), but may be improving developmental conditions, with this species likely to be particularly susceptible to temperature oscillations due to its choice of open or partially shaded breeding sites (Cranston *et al.*, 1987). The effect of Temp30 is less significant ($P<0.05$) than calendar date, suggesting the seasonal abundance pattern for this species is largely stable between years and driven mainly by its diapause timings and univoltinism, with Temp30 influencing adult production during the limited time period it can occur.

Total rainfall 30 days prior to sampling date (Rain30) was retained as a moderately significant ($P<0.01$) predictor for *An. plumbeus* abundance. This species breeds almost exclusively in water-filled tree holes (Cranston *et al.*, 1987; Becker *et al.*, 2010). It was found in these larval habitats in Wytham Woods, where they have a variable volume dependent on rainfall, with evaporation of their free-water possible (Kitching, 1971). Eggs are not laid on the water surface but on the side of the tree-hole, with hatching requiring their immersion (Becker *et al.*, 2010). Thus, higher Rain30 may influence *An. plumbeus* abundance by increasing breeding site availability and inducing higher egg hatch. Higher water volumes are also likely to improve developmental conditions, as larval survival is usually inversely related to density, if diet is limiting (Juliano, 2009). Total rainfall 31-60 days prior to sampling was not retained, suggesting higher rainfall may be influencing *An. plumbeus* abundance mainly by maintaining higher water volume during larval development (which precedes adult emergence). The weaker significance of calendar date ($P<0.1$) in the final *An. plumbeus* model may reflect its apparent bivoltinism, indicating nevertheless moderate between-years stability in timings of higher adult input into the population. Therefore, our results suggest the seasonal abundance pattern of *An. plumbeus* is primarily shaped by its diapause timings and voltinism (as expected for the majority of mosquitoes in temperate climates), but Rain30 exerts an important influence on its preferred breeding sites, likely to induce substantial differences in abundance within and between-years. This will likely render *An. plumbeus* particularly susceptible to reductions in population size due to drought.

Calendar date was not retained for *Cs. annulata*, in accordance with the yearly differences regarding highest abundance month for this species. The absence of significant

environmental predictors may reflect the wide range of larval habitats used by this species, artificial or natural (ponds, cisterns, garden tanks, pools, ponds, marshes, ditches), sunlit or shaded (Marshal, 1938; Medlock *et al.*, 2005), rendering it less susceptible to the effects rainfall and temperature may have on a particular type of breeding site. Therefore, our results suggest *Cs. annulata* has a temporally random abundance pattern between May and September, not significantly influenced by temperature or rainfall, with its seasonal abundance shaped by predictors not considered here. Low abundances and high number of zero catches are likely to have resulted in the substantial lack of fit shown by the GAM models for the remaining species and species groups for whom analysis was attempted (*Cx. pipiens/torrentium*, *Da. geniculata* and *An. claviger*), not allowing an assessment of their relation with the above predictors.

Breeding blue tits were found infected with both *P. circumflexum* and *P. relictum* in early May-early June, with mosquito abundances only increasing sharply in June and decreasing sharply in September. Vector competence studies suggest a temperature dependent period of at least six to ten days, after feeding on infected blood, before a mosquito is able to transmit *P. circumflexum* or P. *relictum* (Valkiūnas, 2005 and references therein, LaPointe *et al.*, 2010). After transmission, a minimum of seven to ten days are required for *Plasmodium* detection in avian host blood, although this period may be shorter for *P. relictum* (Herman, 1938; Valkiūnas, 2005). These periods are likely to increase under non-optimal conditions in the field. Thus, nearly all new infections caused by newly infected mosquitoes in our study area in a calendar year will only be detectable from mid-June onwards. This strongly suggests the prevalence observed in early May-early June must be mostly due to relapse or, alternatively, chronic blood parasitaemia maintained through the

colder months, preceding the majority of *Plasmodium*transmission to susceptible hosts which will necessarily occur during June-August. Furthermore, transmission intensity may increase in the latter part of this period, as the number of older mosquitoes (more likely to feed more than once) may increase in the population. *Plasmodium* was only found in a single mosquito pool in August at the study site (R. Alves, unpublished), providing annedoctical evidence to this assumption. Our results agree with Janovy (1966), who found a spring prevalence peak preceding the appearance of putative vectors in a prairie marsh habitat, and are in accordance with theoretical expectations for avian malaria transmission in temperate regions (Beaudoin *et al.*, 1971).

Our results suggest seasonal patterns of mosquito abundance can remain relatively stable between years in the selected study area (for two of the three species analyzed by GAM). If a similar scenario occurs in the whole study site, we can speculate that the differences in seasonal prevalence between *P. circumflexum* and *P. relictum* reported in Cosgrove *et al.* (2008) are unlikely to be driven by timing of adult vector availability, which will be largely similar between mosquito species and mostly restricted to June-August. The autumn peak seen for *P. circumflexum* did not occur for *P. relictum*, suggesting different transmission intensities during June-August due to differences in vector density, feeding frequency and sporozoite rate (Hay *et al.*, 2000; Kelly-Hope & McKenzie, 2009). A recent study showed that the two avian *Plasmodium* species affect survival and recapture rates differently in blue tits (Lachish *et al.*, 2011a), suggesting avian host-malaria parasite interactions may also play a role in shaping different seasonal patterns of prevalence. Overall, our results support the view that the transmission of mosquito borne disease, such as avian malaria, largely occurs in British woodlands during a limited seasonal interval during the summer.

Furthermore, spring prevalence peaks for avian *Plasmodium* are unlikely to result from transmission earlier in the year. Contrasting year-long transmission intensity and avian malaria prevalence, alongside host-*Plasmodium* interactions, will greatly increase our understanding of the role of vector ecology in shaping seasonal prevalence patterns for this avian disease.

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General Discussion

In this thesis I investigated mosquito-*Plasmodium*-bird associations for avian malaria (*Plasmodium* spp.) in Great Britain and the role of mosquito ecology in shaping these associations in a British woodland site where avian malaria is endemic, using molecular, field ecology and statistical modelling methodologies. In this discussion, the main thesis findings will be summarized and the limitations of this work reviewed, with their relevance discussed both for avian malaria and mosquito research.

Summary of main findings

In chapter 2, molecular genetic techniques were used to investigate avian *Plasmodium* and host associations in 12 British mosquito species or species groups, collected in Oxfordshire woodland and by Somerset agricultural and pasture habitats. The ornithophilic *Cx. pipiens/torrentium* group was found associated with four different avian *Plasmodium* lineages, suggesting an important transmission role for this mosquito group in Great Britain. No other mosquito species or species group was found associated with *Plasmodium*, suggesting the latter may exhibit high vector specificity and be tightly co-evolved with a given vector group. *Cx. pipiens/torrentium* and *Cs. morsitans* were found fed on avian blood in accordance with their known feeding preferences.

In chapter 3, mosquito sampling within a British woodland where avian malaria is endemic, coupled with generalized linear modelling, was used to assess local-scale spatial patterns in abundance of 6 mosquito species or species groups and their relationship with environmental variables. The analysis revealed non-random spatial patterns of abundance and significant environmental predictors, which varied between species or species groups.

Micro-climate, canopy and breeding site variables were all associated with mosquito abundance for at least one species.

In chapter 4, I looked into the association of mosquito density per host (putative vector availability) and landscape variables with *Plasmodium* prevalence for two parasite species (*P. circumflexum* and *P. relictum*), in birds at a local scale. Long-term *P. circumflexum* infection prevalence was strongly predicted by the density per host of *Cs. annulata* (a known experimental vector of this parasite), alongside landscape predictors. Mosquito density per host was not associated with infection prevalence in birds the following calendar year. Overall, landscape variables remained the strongest infection predictors of avian infection prevalence.

In chapter 5, seasonal patterns of mosquito abundance and their associations with temperature and rainfall alongside calendar date were assessed, in a sub-area of the British woodland site used in chapters 3 and 4. Mosquito abundance showed seasonal patterns and was highest between June-August, in general accordance with expectations for temperate climates and previously reported patterns in Great Britain. Average temperature and total rainfall 30 days prior to sampling date were positively associated with *Oc. annulipes* and *An. plumbeus* abundance, respectively, alongside calendar date. Avian *Plasmodium* infection prevalence at the sub-area was assessed for breeding blue tits at a standardized point in their annual cycle (between early May and early June), with a *P. circumflexum* prevalence of 49.1% obtained prior to the seasonal increase in adult mosquito abundance.

Implications for avian malaria transmission and prevalence

The entomological inoculation rate (EIR) is usually used as a measure of transmission intensity and is positively associated with prevalence in human malaria, although this relationship is complex (Beier *et al.*, 1999; Smith *et al.*, 2005). The EIR is a function of mosquito feeding frequency, proportion of mosquitoes with parasites in their salivary glands (sporozoite rate) and mosquito density per host (Hay *et al.*, 2000; Kelly-Hope & McKenzie, 2009). The product of the feeding frequency and mosquito density per host gives the host biting rate (Hay *et al.*, 2000), with human bait catches being the most direct way to measure it in human hosts (WHO, 1975). For this thesis, assessing the biting rate in wild conditions would require entrapment of avian hosts of particular interest (blue tits and great tits) and their maintenance in captivity for a period encompassing at least two hours before sunset and after sunrise (the period of highest mosquito activity for most species). Assessment of the feeding frequency in laboratory conditions would require birds to be caged for periods encompassing mosquito longevity, which can exceed six weeks (Service *et al.*, 1980). Experimental infection studies looking into the vector competence for *P. circumflexum* and *P. relictum* of the mosquito fauna found could provide proof of vector competence, but would also require caging of suitable avian hosts. Hence, whilst such methodologies could be expected to enhance our knowledge regarding avian malaria transmission in our main study site and for British mosquitoes in general, they are also logistically difficult to implement and raise ethical challenges (e.g. when requiring taking wild birds into captivity), and were not pursued. Attempts to assess sporozoite rate were made mostly indirectly (on thoracic body parts), but also directly on mosquito salivary glands. Malaria parasites were only found in *Cx. pipiens*/*torrentium* abdominal body parts

and at the very low infection rates (Chapter 2) reported by other authors (see Chapter 1). An inefficient sampling methodology for mosquitoes fed on birds is a possible issue in this study; *Plasmodium* absence in thoraxes or salivary glands may be due to very low parasite number or arrested development. Therefore, assessment of sporozoite rates was not feasible. The comprehensive sampling at a spatial scale of avian hosts and mosquitoes in Wytham Woods allowed me to assess mosquito density per host, which was strongly associated with long-term spatial *P. circumflexum* prevalence in the case of *Cs. annulata*, a known experimental vector of this parasite (Chapter 4). Comprehensive sampling of avian hosts at a seasonal scale was undertaken only during a standardized point in their annual cycle, between early May and early June (Chapter 5). Therefore, the overall findings in this thesis do not allow for estimates of EIR (both at a spatial or temporal scale) and how they may associate with avian malaria prevalence, but provide insights regarding its transmission and the identity of likely vectors.

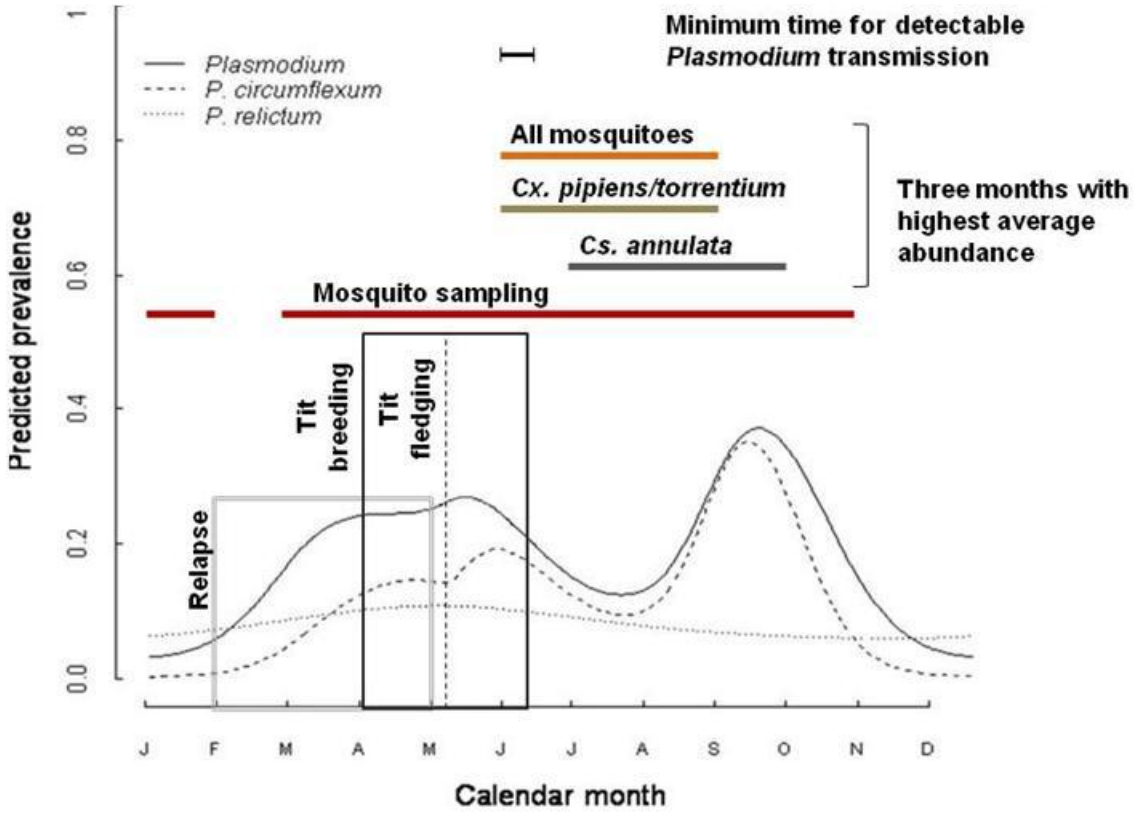
First, findings here suggest mosquito density per host as a variable capable of influencing spatial patterns of avian malaria prevalence, according to theoretical expectations for malaria transmission (MacDonald, 1957), even at a local scale and within a single host population. Hence, the study of avian malaria will benefit from considering this variable when possible at such fine spatial scales. Furthermore, mosquito density per host can be expected to inform more about the role of mosquito ecology in avian malaria than mosquito abundance, as it takes in consideration host density. Nevertheless, mosquito density per host only predicted long-term prevalence and only for *P. circumflexum*. Therefore, mosquito density alone cannot explain avian *Plasmodium* prevalence, highlighting the need to also assess the other components of the EIR (feeding frequency and sporozoite rate) to

understand transmission of these parasites. Furthermore, EIR must be considered alongside avian host, malaria parasite and environmental variables to understand the prevalence and evolution of this avian disease.

Second, findings presented here suggest that transmission of mosquito borne disease in British woodlands occurs mostly during a seasonally limited period (June-August), which for avian malaria is likely to follow the occurrence of a spring relapse of previously acquired infections or, alternatively, chronic blood parasitaemia maintained through the colder months (see Figure 6.1 for relevant timings of mosquito abundance, blue tit life cycle and *Plasmodium* prevalence and putative relapse in birds). This agrees with Janovy (1966), who found a spring prevalence peak for avian malaria preceding the appearance of putative vectors in a temperate prairie marsh habitat, further confirming theoretical expectations for avian malaria transmission in temperate regions (Beaudoin *et al.*, 1971) and providing additional evidence that the study of avian malaria prevalence in these habitats will greatly benefit from considering EIR parameters from previous calendar years.

Third, my results suggest *Cx. pipiens/torrentium* has a vector role in Great Britain for *P. relictum* and possibly other *Plasmodium* morphospecies, while *Cs. annulata* may vector *P. circumflexum*. For *Cx. pipiens/torrentium*, a vector role is strongly suggested by molecular analysis (Chapter 2) and is in accordance (for *Cx. pipiens*) with other studies looking into mosquito-*Plasmodium* associations in European habitats (Glaizot *et al*., 2012; Ventim *et al*., 2012), and with experimental infections in laboratory conditions using European mosquito strains (Valkiūnas, 2005 and references therein; Vézilier *et al*., 2010). Also, *Cx. pipiens/torrentium* was found fed on avian blood (Chapter 2) in accordance with its known feeding preferences in Great Britain (Service, 1969; Service, 1971), further suggesting a

Figure 6.1 - Overview of timings relevant for assessment of seasonal mosquito borne disease transmission in British woodlands. Data for *Plasmodium* prevalence in avian hosts, blue tit life cycle and mosquito abundance was obtained in Wytham Woods (Oxford, United Kingdom) and is described in more detail in Chapters 1 and 5, and in Cosgrove *et al*. (2008). "Mosquito sampling" corresponds to the seasonal period mosquito abundance was assessed in Wytham Woods (Chapter 5). Timing of putative *Plasmodium* "Relapse" was obtained from Applegate *et al*. (1971) (experimental work with English sparrows elsewhere) and "Minimum time for detectable *Plasmodium* transmission" (short black bar) corresponds to the minumum period required, under optimal conditions, between a mosquito blood meal in an infected avian host and detection of *Plasmodium* in the blood of another avian host, following a second blood meal from the same mosquito (experimental work elsewhere, summarized in Valkiūnas, 2005) (figure adapted from Cosgrove *et al*. (2008) and Chapter 1).



possible vector role in the wild in Great Britain. Several avian *Plasmodium* lineages, not yet assigned to a morphospecies, were also found in *Cx.pipiens*/*torrentium*, implying avian *Plasmodium* transmission in Great Britain may be mediated mainly by this mosquito group. This possibility agrees with other studies in European habitats (Huijben *et al*., 2007; Glaizot *et al*., 2012), who found avian *Plasmodium* associated with *Cx. pipiens* only. Hence, results here further suggest avian *Plasmodium* may present high vector specificity and tight co-evolution with a given mosquito species, in contrast with the scenario proposed in other geographic regions, where several mosquito species are usually found associated with these malaria parasites, which show low vector specificity (e.g. Ishtiaq *et al*., 2008; Njabo *et al*., 2011). Therefore, the study of *Cx.pipiens*/*torrentium* associations with avian *Plasmodium* using British strains may provide valuable insights into mechanisms of vector specificity and co-evolution. For *Cs. annulata*, a possible vector role in Great Britain is suggested by the strong spatial association found between its density per host and *P. circumflexum* prevalence, a possibility backed by its confirmed experimental vector status for this malaria parasite elsewhere (Reichenow, 1932). Given this association, it may appear surprising that *P. circumflexum* was not detected in *Cs. annulata* by PCR; however, this may be due to low numbers of mosquitoes processed. The most abundant mosquito species in Wytham Woods was *Oc. annulipes*. This mosquito species did not harbour detectable *Plasmodium*, was found fed only in mammalian blood and results here do not suggest its density per host was related to avian malaria prevalence in tits in Wytham. Hence, evidence suggests the most abundant mosquito species found at an endemic avian malaria site does not vector avian *Plasmodium*, highlighting the need to consider the whole mosquito fauna when studying the transmission of this disease.

Experimental studies are desirable to provide definitive proof regarding which British mosquito species or species groups are able to vector avian *Plasmodium*, with assessment of feeding preferences confirming a role in the wild as vectors for bird species of interest.

Implications for mosquito ecology

Geomorphology, climate and landscape anthropogenic variables (e.g. artificial breeding sites such as containers or dams) combine to form spatial structure in ecosystems, which vary in their capacity to support mosquitoes (Reisen *et al*., 1990; Reisen, 2010). At a seasonal scale, temperature and rainfall are regarded as the main environmental variables influencing mosquito ecology (Hoshen & Morse, 2004). Knowledge regarding spatiotemporal variation in mosquito abundance and its environmental predictors informs the study of mosquito-borne disease and the implementation of effective surveillance and control methodologies, a matter of particular importance in a world of climatic and landscape changes. In this thesis, insights were obtained regarding mosquito abundance patterns and their environmental predictors within a British woodland, where avian malaria is known to be endemic.

To my knowledge, this is the first study formally testing the occurrence of non-random spatial patterns of mosquito abundance at a local scale in British woodland, showing these can occur and vary between species or species groups. This agrees with findings elsewhere, with mosquito abundance known to vary at small geographic scales in humanized areas (Cano *et al*., 2006; Reiter & LaPointe, 2007) and less humanized landscapes such as wetlands (Gleiser *et al*., 2002; Schäfer *et al*., 2006) or semiarid plains (Barker *et al*., 2009). Hence, the study of British woodland mosquito populations will also benefit from

considering the possibility of local-scale spatial heterogeneity in abundance. The seasonal abundance patterns found differed between species or species groups and generally agree with findings elsewhere in Great Britain (Cranston *et al*., 1987; Medlock *et al*., 2005; Hutchison *et al.*, 2007; Snow & Medlock, 2008; Clarkson & Setzkorn, 2011), being also in accordance with typical patterns for temperate climates (Altizer *et al.*, 2006). Calendar date was a predictor of mosquito abundance in this multi-year study, in agreement with known innate diapausing behaviors that primarily shape seasonal abundance patterns, rendering June-August as the period of highest mosquito abundance for the majority of British mosquitoes (Cranston *et al.*, 1987). Hence, this study provides further evidence that mosquito research in Great Britain must consider the period encompassing June-August, and expect seasonal differences between species.

Several environmental predictors were found for mosquito abundance at a spatial and seasonal scale within-woodland. Climatic (temperature, rainfall, humidity) and landscape (canopy height and distances to breeding sites) variables were included in predictor suites, which varied markedly between species or species groups. This is in agreement with a scenario of pronounced differences in ecological requirements, which typically occurs between sympatric mosquito species (Service, 1980; Becker, 2010). Strong support for a predictor role seldom occurred for a given environmental variable, both at a spatial and seasonal scale. Furthermore, the explanatory power of the final models obtained for mosquito abundance was generally low (except for *Oc. annulipes*). This may be due to unaccounted predictors such as host abundance, temporary water pools or water-filled tree-holes, which may exhibit spatial and seasonal variation within and near to the study site likely to influence mosquito abundance. Moreover, mosquito collections were relatively

low for the majority of species or species groups (compared with *Oc. annulipes*), with stronger data robustness (more records/samples processed) likely to allow for stronger insights into abundance predictors. Overall, results here suggest a complex set of predictors is driving mosquito abundance in British woodlands, with their influence likely to shape mosquito ecology even at small geographic scales. The study of mosquito ecology and epidemiological role in British woodlands, including the design of effective control and monitoring tools, must therefore take in consideration this complexity and avoid simplistic predictions. Such studies should consider landscape variables, including distance to breeding sites and canopy height, and climatic variables, including temperature, humidity and rainfall, as candidate predictors of the abundance of British woodland mosquitoes.

Further work

Stronger evidence is needed to fully establish the vectors of avian malaria in Great Britainin the wild. Screening of mosquito salivary glands is a strong methodology to assess vector role, but experimental infections with suitable avian hosts are desirable to fully establish vector competence (Njabo *et al*., 2009; Njabo *et al*., 2011). Experimental infections also have the potential to inform about mosquito feeding rate, alongside behavioral changes (Schwartz & Koella, 2001) and fitness costs (Ferguson *et al*., 2003) of harboring avian *Plasmodium*. Hence, experimental infection has the potential to provide essential data that on British mosquito-avian *Plasmodium* associations that cannot easily be derived by other approaches. It is therefore desirable that the logistical and ethical challenges experimental

infections raise can be overcome. For example, by establishing insectaries with wild-caught British mosquitoes coupled with the use of informative and domesticated avian hosts).

The body of work undertaken with the avian malaria system in Wytham Woods highlighted *Cs. annulata* density per host (Chapter 4) and distance to the nearby river Thames (Wood et al., 2007; S. Lachish *et al*., 2012, Chapter 4) as the main determinants of *P. circumflexum* spatial prevalence in tits. *Cs. annulata* abundance was relatively low (7.6 % of collections, Chapter 3) and the river is unlikely to act as a main breeding site for mosquitoes (Cranston *et al*., 1987; Becker *et al*., 2010), suggesting other factors may contribute directly to the striking *P. circumflexum* spatial prevalence pattern. Avian host immunity may be an important factor here, with environmental variables hypothetically inducing differences in immunological response (Moullac & Haffner, 2000; Cheville, 1979) to *P. circumflexum* in tits. Hence, the study of avian host immunity alongside infection prevalence, mosquito, environmental and other host variables may prove to be a fruitful area of research in Wytham Woods.

General Conclusions

This thesis provided the first analysis of mosquito-*Plasmodium* associations in the wild for avian malaria in Great Britain. Furthermore, it conducted a fine-scale assessment of spatial and seasonal mosquito abundance, alongside an analysis of their sources of variation, within a British woodland site where avian malaria is endemic. I showed that the *Cx.pipiens*/*torrentium* group is likely to have a main role in avian malaria transmission in Great Britain, while *Cs. annulata* may be transmitting *P. circumflexum*. I also demonstrated

an association between mosquito density per host and avian malaria prevalence for *Cs. annulata*, and that transmission of avian *Plasmodium* in British woodlands is likely to be limited mainly to June-August. This thesis also showed that local-scale spatial heterogeneity in mosquito abundance occurs within British woodlands, varying between species or species groups; furthermore, both spatial variation and seasonal abundance are likely driven by a complex set of environmental variables. The fine-scale heterogeneity in mosquito parameters found within an avian malaria endemic site highlights the need to anticipate this complexity when trying to understand avian malaria transmission. By doing so, we further extend the potential of avian malaria systems to improve our knowledge regarding the ecology and evolution of host-parasite-vector systems.

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Molecular epidemiology of malaria prevalence and parasitaemia in a wild bird population

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Abstract

Avian malaria (*Plasmodium* spp.) and other blood parasitic infections of birds constitute increasingly popular model systems in ecological and evolutionary host–parasite studies. Field studies of these parasites commonly use two traits in hypothesis testing: infection status (or prevalence at the population level) and parasitaemia, yet the causes of variation in these traits remain poorly understood. Here, we use quantitative PCR to investigate fine-scale environmental and host predictors of malaria infection status and parasitaemia in a large 4-year data set from a well-characterized population of blue tits (*Cyanistes caeruleus*). We also examine the temporal dynamics of both traits within individuals. Both infection status and parasitaemia showed marked temporal and spatial variation within this population. However, spatiotemporal patterns of prevalence and parasitaemia were non-parallel, suggesting that different biological processes underpin variation in these two traits at this scale. Infection probability and parasitaemia both increased with host age, and parasitaemia was higher in individuals investing more in reproduction (those with larger clutch sizes). Several local environmental characteristics predicted parasitaemia, including food availability, altitude, and distance from the woodland edge. Although infection status and parasitaemia were somewhat repeatable within individuals, infections were clearly dynamic: patent infections frequently disappeared from the bloodstream, with up to 26% being lost between years, and parasitaemia also fluctuated within individuals across years in a pattern that mirrored annual population-level changes. Overall, these findings highlight the ecological complexity of avian malaria infections in natural populations, while providing valuable insight into the fundamental biology of this system that will increase its utility as a model host–parasite system.

Keywords: blue tit, haemosporidian, host–parasite interactions, *Plasmodium*, qPCR

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Introduction

Understanding the causes of host variation in susceptibility to infectious disease is central to understanding and predicting disease dynamics and host–parasite evolution. Yet for many diseases of wild animal populations, such fundamental knowledge remains limited. Whether an individual becomes infected by a given parasite will depend on environmental factors that influence exposure, such as climatic effects on vector or parasite development (Rogers & Randolph 2006), as well as intrinsic host factors such as genetics (Kaslow *et al*. 2008), permanent environmental effects (Monaghan 2008) or effects of host age on immunity (Palacios *et al*. 2007). Thus, the distribution of parasites among hosts is likely to be shaped by a combination of abiotic and biotic factors, and so understanding their relative contributions is an important prerequisite for predicting the evolution of host–parasite relationships. From a

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practical perspective, understanding the sources of variation in parasitological traits is also important so that hypotheses can be framed and tested appropriately. For instance, if parasite prevalence shows strong temporal or spatial variation within populations, conclusions from cross-population comparisons may be strongly influenced by chance decisions about when or where to sample.

Since the development of molecular screening techniques for avian haemosporidia, these parasites have become an increasingly popular model system for testing hypotheses about host–parasite interactions in wild birds (Bensch *et al.* 2009). Numerous studies have documented extensive and complex variation in the prevalence and diversity of these common vector-borne parasites in both vertebrate hosts and mosquitoes, between geographically separate host populations and across biogeographical regions (Bensch & Åkesson 2003; Santiago-Alarcon *et al.* 2008; Chasar *et al.* 2009; Njabo *et al.* 2011). Within-population variation in prevalence according to various abiotic and host factors has also been reported, if less frequently (Bensch *et al.* 2007; Wood *et al.* 2007). However, the biological processes that underpin such prevalence variation remain poorly understood. Moreover, although prevalence (or infection status at the individual level) can reveal whether an organism has become infected or not, this trait offers little insight into host–parasite interactions after the point of infection. Parasitaemia (the density of parasites within infected hosts), although less often measured, may be more informative in this respect: whereas infection status may be determined by the degree of exposure to infective vectors (Sol *et al.* 2000) or intrinsic host factors such as genetic resistance (Westerdahl 2007), parasitaemia may reflect hosts' ability to control established infections, or variation in parasite replication rate in response to a variable host environment (Reece *et al.* 2009). Furthermore, as parasitaemia often influences the probability of detecting infections (Bentz *et al.* 2006; Fallon & Ricklefs 2008), the extent to which documented patterns of prevalence variation may be driven by variation in parasitaemia is currently unclear. Thus, studies that tease apart these two traits in wild populations are critical to further our understanding of avian malaria ecology. For true avian malaria parasites (*Plasmodium*), quantitative data on parasitaemia from field studies are scarce. A major reason for this is the difficulty of measuring this trait, as in *Plasmodium* infections of wild-caught birds, parasite densities are often too low for accurate microscopic quantification (Fallon & Ricklefs 2008). However, with the adoption of quantitative PCR for measuring parasitaemia, the first data on this trait from wild hosts are now emerging (Bentz *et al.* 2006; Zehtindjiev *et al.* 2008).

A further limitation to our understanding of avian malaria ecology is the paucity of longitudinal data on within-individual infection dynamics, particularly in natural settings. As wild birds are rarely resampled, little is known about how temporally stable or dynamic chronic infections are over time. It has been assumed that infection status measured at a single time point can reflect host resistance or susceptibility to malaria, and associations have been sought between infection status and MHC genotype as well as overall genetic diversity on this basis (Ortego *et al.* 2007; Westerdahl 2007; Loiseau *et al.* 2008). However, the value of such an approach will depend on the temporal stability of individual infection status and how this relates to genetic resistance, as well as the extent to which the compared hosts have been equally exposed to infection. Longitudinal data can also provide insight into the biological processes that give rise to observed cross-sectional patterns in parasitological traits. For example, a frequently reported cross-sectional pattern among studies of avian blood parasites is an increase in prevalence across host age cohorts, which is thought to reflect an increased duration of exposure to parasites among older individuals, and maintenance of chronic infections (Stjernman 2004; Wood *et al.* 2007). However, several non-exclusive processes, including age-specific probabilities of infection gain or loss and infection-related mortality could be involved, and without longitudinal analyses these cannot be teased apart (Sol *et al.* 2003).

Here, we present results from a 4-year longitudinal study on the epidemiology of *Plasmodium* infections in a woodland population of blue tits (*Cyanistes caeruleus*). Using a newly developed qPCR assay to measure parasitaemia and a combination of cross-sectional and longitudinal approaches, we conduct a detailed analysis of environmental and host factors predicting variation in both infection status and parasitaemia in this population, and the variability of these traits within individuals over time.

Methods

Field methods

The study was conducted in Wytham Woods (51°46′N, 1°20′W), a 385-ha mixed deciduous woodland near Oxford, UK, where 250–450 pairs of blue tits breed in nestboxes every year (Perrins 1979). Each breeding season (April–June), all 1205 nestboxes at the site are monitored at regular intervals, so that lay date (date on which the first egg was laid; LD), hatch date (date on which the first egg hatched; HD) and clutch size (CS) were determined for all reproductive attempts. The analyses conducted here involve samples collected from

breeding adults between 2005 and 2008. Adults were captured and blood-sampled when their broods were between 6 and 15 days post-hatch. In this way, all hosts were sampled at a standardized point in their annual cycle, thus controlling for previously documented seasonal variation in parasite prevalence at this location (Cosgrove *et al.* 2008). All blood samples were taken by brachial or jugular venepucture, under Home Office licence. In 2006 and 2007 as part of another experiment (Knowles *et al.* 2010a), a subset of females were additionally sampled around egg-hatching, thus providing two samples per year approximately 2 weeks apart for these individuals. Host sex was determined based on the presence (female) or absence (male) of a brood patch, and age (first year or older) according to plumage characteristics (Svensson 1992). For 75% of the individuals in this study, an exact age could be assigned, because they were either first captured as yearlings, or born locally and ringed as nestlings. For non-local birds first captured with adult plumage, a minimum age of two was assigned. Thus, all birds in the population could be assigned a minimum age at each capture.

Avian malaria diagnosis

We used quantitative (q)PCR for detecting and quantifying *Plasmodium* parasites, using the primers L9 5′-AAACAATTCCTAACAAAACAGC-3′ and NewR 5′-ACATCCAATCCATAATAAAGCA-3′, which target a 188-bp region of the mitochondrial cytochrome *b* gene. Genomic DNA was extracted from blood samples, using DNeasy extraction kits (Qiagen) for samples from 2005, and a standard ammonium acetate method for samples from 2006 to 2008. DNA concentration was measured using a Picogreen assay (Quant-iT Picogreen dsDNA Assay Kit, Invitrogen) and samples were diluted to a working concentration of 2 ng/μL prior to qPCR. Standard curves were created using a full-length cyt *b* PCR product from *P. relictum* (lineage pSGS1) amplified using the protocol of Perkins & Schall (2002). qPCR reactions were performed exactly as described in Knowles *et al.* (2010b). For the purpose of analysing within-individual changes in parasitaemia, all samples for a given individual were run on the same qPCR plate, to eliminate the possibility of artefactual effects arising because samples were tested in different batches.

Nested PCR (Waldenström *et al.* 2004) was also performed on samples from 2005 to 2007 as described elsewhere (Wood *et al.* 2007), allowing us to compare the detection sensitivity of our qPCR assay with that of a commonly used non-quantitative PCR assay. In addition, nested PCR sequence data was used to identify a consistent difference in qPCR product melting temperature that could be used in *Plasmodium* species diagnosis. Two divergent cyt *b* clades were regularly detected in this population (comprising 98.2% all infections), which correspond to two well-defined morphospecies, *Plasmodium relictum* (lineages pSGS1 and pGRW11) and *P. circumflexum* (lineages pTURDUS1 and pBT7; Palinauskas *et al.* 2007). For simplicity, we refer to them by their morphospecies classification from here on. Because qPCR products from *P. relictum* melted around 1 °C above those from *P. circumflexum* (approximately 75.2 °C vs. 74.2 °C), we used qPCR product melting temperature to diagnose *Plasmodium* species (see Data S1 in Supporting information for further details).

To estimate the repeatability of qPCR parasitaemia estimates, DNA was re-extracted from 35 blood samples from 2008 and qPCR repeated as described earlier. ln(1 + *Plasmodium* DNA copies), our measure of parasitaemia in analyses, was highly repeatable between extractions, with $r = 0.80$ among samples where at least one tested positive ($n = 28$). For a larger number of samples ($n = 360$), qPCR was repeated on the same DNA extraction and revealed similar levels of repeatability ($r = 0.78$; $n = 193$); this data set was also used to ascertain the probability of non-detection for samples known to be positive, and the rate at which prevalence increases when the same samples are tested repeatedly (see Results).

Measurement of breeding location and environmental variables

To investigate broad spatial trends in prevalence and parasitaemia across the study site, we used GIS-derived measures of the position of each nestbox where a blue tit was captured and sampled. The x and y coordinates of all nestboxes in Wytham were known (±3 m), as these were digitally mapped using differential GPS in 2004–2005 (Wilkin *et al.* 2007a). These coordinates and their interaction term were entered in cross-sectional analyses of infection status and parasitaemia to test for linear effects of nestbox location ('latitude' and 'longitude') as well as broad patterns of spatial clustering for these traits within the woodland. As breeding tits are territorial and forage in the immediate vicinity of the nest (Stauss *et al.* 2005), these coordinates give an accurate description of individuals' location at this time of year. As nestbox coordinates predicted both prevalence and parasitaemia, we created interpolated maps in MapInfo professional v8.5 to visualize spatial variation in these traits with greater resolution. Interpolations were created using inverse distance weighting (IDW), which uses a moving point average to interpolate pixel values and estimate local trends in spatially variable data. Each pixel value is calculated by averaging the

weighted sums of all data points within a user-defined search area, such that points farther away influence the pixel value less than those that are close (decay exponent = 2). The radii of the search and display areas used were four times the average point density (400 m) as in Wood *et al.* (2007). Environmental predictors of spatial variation in prevalence in this population have been documented previously (Wood *et al.* 2007), and so we do not explore these further here. However, we explored possible environmental drivers of spatial variation in parasitaemia, using four variables that describe the local environment of a nestbox, derived using GIS software (MapInfo Professional v7.8 & Vertical Mapper v.3):

1. *Territory size.* Tessellations (Thiessen polygons) were formed around nestboxes by placing boundary lines equidistant between occupied nestboxes in each year. Polygon area (ha) is inversely related to breeding density and provides an individual-specific measure of territory size (Wilkin *et al.* 2006). As great tit territories are interspersed with those of blue tits and these species share similar breeding ecology and are known to compete (Minot & Perrins 1986), we included great tit breeding events when calculating territory size. As the distribution of territory sizes was heavily right-skewed, we used a log-transformation in analyses (which was approximately normally distributed) to reduce the potential for extreme values to heavily influence results.
2. *Oak tree abundance.* In addition to nestboxes, the locations of all mature pedunculate and sessile oak trees (*Quercus robur* and *Q. petraea*) in Wytham were mapped due to their importance for tit breeding ecology; caterpillars of species such as the winter moth (*Operophtera brumata*) constitute a primary food source for tits during the breeding season (Wilkin *et al.* 2009) and occur most abundantly on newly emerged oak leaves (Feeny 1970). We therefore used the number of oaks within a 50 m radius of each nestbox (Oaks50) as a measure of local food availability (Wilkin *et al.* 2009).
3. *Proximity to the woodland edge.* We used an edge distance index (EDI; defined in Wilkin *et al.* 2007b) as a measure of how peripheral a nestbox is at the study site. EDI accounts for both the edge distance and the number and layout of edges near a nestbox. Environmental quality is expected to increase with EDI (Wilkin *et al.* 2007b).
4. *Altitude.* As Wytham covers a low-lying hill, the altitude of nestboxes ranges from approximately 60 to 160 m and is inversely correlated with edge distance (Wilkin *et al.* 2007b). Because several potentially relevant habitat features vary altitudinally (e.g. temperature, soil type and water content) and in order to distinguish between edge and altitude effects, we included nestbox altitude as well as an interaction between altitude and EDI in environmental analyses.

These nestbox-specific measures of territory size, oak abundance, altitude and EDI are all associated with either life-history traits or measures of reproductive success for great tits at the study site (Wilkin *et al.* 2006, 2007a, 2009), which are similar in their breeding ecology to blue tits, and therefore reflect important components of the local environment during breeding. Because *Plasmodium* infections seem to be acquired in the Summer/Autumn after birds have settled on their breeding territories in this population (Cosgrove *et al.* 2008; S. Knowles unpublished data), these measures provide a description of individuals' local environment around the time of malaria transmission as well as during breeding.

Statistical analyses

Cross-sectional analyses of infection status. Infection status was modelled using binomial generalized linear models (GLMs) with a logit link, both for pooled *Plasmodium* infections as well as *P. relictum* and *P. circumflexum* separately. These analyses included only samples collected from breeding blue tits 6–15 days after their first egg hatched. Where multiple years' data were available for an individual, all but one was randomly excluded from analysis. We tested for effects of five host factors on infection status: minimum age, a quadratic age term (minimum age^2), sex and two reproductive parameters—clutch size (CS) and standardized hatch date (HD_{std}). HD_{std} measures an individual's relative timing of breeding (and therefore also sampling) within a given year, and is calculated as (HD—annual mean HD)/annual HD standard deviation. To test for spatial variation in infection probability, nestbox x and y coordinates were included as well as their interaction term. Because several effects on infection status appeared to differ between *Plasmodium* species, we then used a binomial generalized linear mixed model (GLMM) to test directly for species differences in infection status predictors. In this model, each sample was represented by two data points: one diagnosis for *P. relictum* and one for *P. circumflexum*. Multiple samples per individual (where available) were included in this analysis, which allowed us to test the robustness of results from previous analyses that excluded all but one datapoint per individual. All effects that predicted infection status for either species were included in the starting model, as

well as all two-way interactions with species. To control for pseudoreplication arising from this approach (each sample represented twice, and some individuals multiple times), we included sample identity nested within individual identity as a random effect in this analysis.

Longitudinal analyses of infection status. To investigate the temporal stability of infection status, we examined three parameters, across three time intervals. These parameters were (i) diagnostic agreement rate (the proportion of identical diagnoses) (ii) the probability of infection loss for individuals or samples first scored as *Plasmodium*-positive; (iii) the probability of infection gain for individuals or samples first scored as *Plasmodium*-negative. The first time interval relates to the same DNA extraction being tested twice ($n = 358$ samples) and serves as a control to estimate apparent loss and gain because of diagnostic error. The second interval concerns females tested approximately 2 weeks apart during the same breeding season, once around hatching and once whilst feeding young ($n = 227$ females, mean = 13.0 ± 0.14 days interval, range 5–22 days). The third interval concerns individuals tested whilst feeding young in separate years ($n = 238$ individuals, including both males and females). For between-year analyses, only the first pair of years where the given parameter could be estimated were used for each individual; in most cases, this related to a 1-year interval (e.g. in the diagnostic agreement rate analysis, 1-year interval $n = 224$, 2-year $n = 32$, 3-year $n = 5$). By comparing loss and gain rates across these intervals, one can test for real loss and gain of infection above detection failure and assess the timeframe over which such processes occur. Infection gains and losses were estimated both for pooled *Plasmodium* and on a species-specific basis; thus, a sample that switched *Plasmodium* species between time points was treated as maintenance of overall *Plasmodium* infection in the pooled analysis, but as a loss of one species and gain of another in species-specific analyses.

Parasitaemia analyses. As the distribution *Plasmodium* cyt *b* copy number was heavily right-skewed, to meet GLM assumptions we modelled parasitaemia as ln(1 + *Plasmodium* copies), which was approximately normally distributed. Parasitaemia was modelled using GLMs with a normal error structure and an identity link function, including in the starting model the same predictor variables tested for infection status, together with all 2-way interactions with *Plasmodium* species. For individuals present multiple times in this data set, all but one entry was randomly excluded. To explore spatial effects on parasitaemia further, we tested for effects of territory size, EDI, altitude and Oaks50 (see above) on parasitaemia in a further GLM, including all significant effects from the original model for parasitaemia, except nestbox coordinates. Repeatability of parasitaemia measurements was estimated using data from individuals *Plasmodium*-positive more than once, across the same three time intervals as described earlier.

All models were simplified by backwards stepwise elimination, sequentially removing terms for which $P > 0.1$ and finally those with $P > 0.05$ to leave the minimal model. GLMs were performed in JMP v.6 (SAS Institute, 2005), and GLMMs using the GLIMMIX platform in SAS v9.2 (SAS Institute, Cary, NC), using the Kenward-Roger approximation for degrees of freedom.

Results

Assay sensitivity and comparison of nested and qPCR

Agreement in diagnosis by qPCR and nested PCR was relatively high, with infection status by one method strongly predicting status by the other (agreement rate = 78%, $\chi^2_1 = 36.80 = 428.44$, $P < 0.001$, $n = 1254$). However, the sensitivity of qPCR detection was significantly higher (Table S1 in Supporting information). Among samples positive by qPCR, parasitaemia positively predicted detection probability by nested PCR ($\chi^2_1 = 191.42$, $P < 0.001$, $n = 548$). Similarly, among samples tested twice by qPCR, the probability of detection on the second test was positively predicted by initial parasitaemia ($\chi^2_1 = 36.80$, $P < 0.001$, $n = 161$). Thus, for both methods, samples with higher parasitaemia were more likely to be detected. All results presented from here on concern qPCR diagnoses only. (See Data S1 and Fig. S1 in Supporting information for further details of assay sensitivity and parasite diversity detected by nested PCR).

Cross-sectional analyses of infection status

Overall *Plasmodium* prevalence was 42.0%, with 22.6% of individuals infected by *P. relictum* and 19.1% by *P. circumflexum*. Results from cross-sectional analyses of factors predicting infection status are shown in Tables 1 and S2 (Supporting information). Prevalence varied markedly across the 4 years studied, and these annual fluctuations differed between *Plasmodium* species, with *P. relictum* exhibiting more pronounced annual fluctuations and *P. circumflexum* prevalence more stable across years [Tables 1 and S2 (Supporting information); Fig. 1a, b]. When only yearling birds were considered (i.e. only individuals that could have acquired infection within the preceding year), prevalence patterns for both species closely matched those including all individuals (Fig. 1a, b). Nestbox location also strongly predicted

Appendix 1

Table 1 Factors predicting malaria infection status (prevalence) in the blue tit population of Wytham Woods

		All *Plasmodium* (n = 1499)			*P. relictum* (n = 1496)			*P. circumflexum* (n = 1496)		
Predictor	df	χ^2	P	r	χ^2	P	r	χ^2	P	r
Minimum age	1	16.78	**<0.001**	**0.106**	6.05	**0.014**	**0.064**	4.81	**0.028**	**0.057**
Minimum age^2	1	4.44	**0.035**	**−0.054**	1.76	0.184	−0.034	0.73	0.394	−0.022
Year	3	25.04	**<0.001**		66.67	**<0.001**		11.02	**0.012**	
x	1	13.22	**<0.001**	**−0.094**	0.14	0.711	0.010	15.61	**<0.001**	**−0.102**
y	1	3.44	**0.064**	**0.048**	31.13	**<0.001**	**−0.144**	75.09	**<0.001**	**0.224**
x*y	1	6.63	**0.010**	**−0.067**	0.34	0.560	0.015	3.28	0.070	−0.047
Sex (F)	1	0.73	0.394	0.022	4.21	**0.040**	**0.053**	0.37	0.544	−0.016
Clutch size	1	1.80	0.1780	0.035	0.83	0.363	0.024	0.36	0.552	0.015
HD_{std}	1	0.62	0.431	0.020	0.81	0.369	0.023	1.39	0.239	−0.031

All variables in the starting model are listed, with effects in the minimal model in bold. For significant effects ($P < 0.05$), statistics are from the minimal model; for non-significant effects, statistics are presented from the last model that included this term. Effect sizes (Pearson's r) are given for all effects with a single degree of freedom.
HD_{std}, standardized hatch date, calculated as described in methods; x and y are the geographical coordinates of an individual's nestbox.

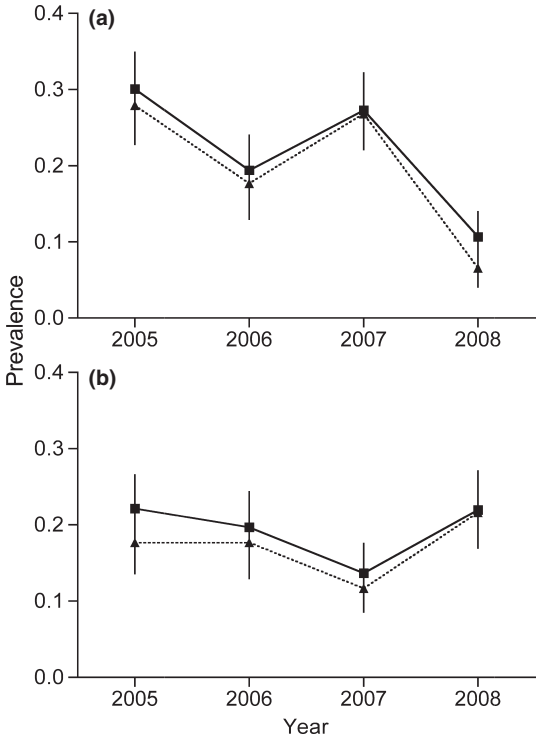

Fig. 1 Annual changes in prevalence for (a) *Plasmodium relictum* and (b) *P. circumflexum*, for all birds (squares) and yearlings only (triangles). Means and 95% confidence intervals are plotted using raw data.

infection status, and again patterns were species-specific. Although latitude (y coordinate) predicted infection status for both species, the effects were in opposite directions, with *P. circumflexum* more prevalent in the north and *P. relictum* in the south of the woodland; in addition, whilst *P. circumflexum* was more prevalent in the west, there was no significant effect of longitude for *P. relictum* [Tables 1 and S2 (Supporting information); Fig. 3a–c]. To explore whether these spatial patterns were broadly stable across years, we tested for year interaction terms with x, y or x*y in the minimal model for each species. None of these year interaction terms were significant (*P. relictum*: year*y $\chi^2_1 = 1.71$ $P = 0.634$; *P. circumflexum*: year*x $\chi^2_1 = 0.59$, $P = 0.899$, year*y $\chi^2_1 = 5.20$, $P = 0.158$, year*x*y $\chi^2_1 = 1.45$, $P = 0.766$), suggesting a degree of spatial stability in the infection patterns. For *P. relictum* (but not *P. circumflexum*), we found some evidence that females were more likely to be infected [Tables 1 and S2 (Supporting information)]. Neither clutch size nor hatch date (HD_{std}) predicted infection status for either species (Table 1).

Malaria prevalence showed a significant quadratic relationship with host age, increasing initially but declining among older hosts (Table 1, Fig. 2a), a pattern that was similar for both *Plasmodium* species (Table S2 in Supporting information). To examine which processes might underlie this pattern, we calculated the expected age-prevalence pattern under a simple set of assumptions (scenario 1). These assumptions were (i) all infections are maintained and cannot be lost; (ii) the risk of becoming infected is constant (e.g. regardless of age or year) and equals the prevalence among yearling birds; (iii) mortality, recapture probability and dispersal of birds into or out of the population are all independent of infection status and age. The observed age-prevalence pattern clearly differs from this scenario (Fig. 1a) and a goodness of fit test, in which the age distribution of infected individuals is

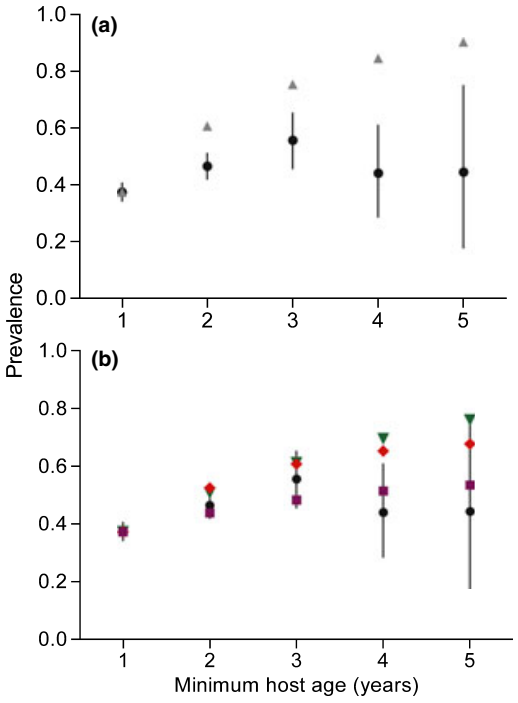

Fig. 2 (a) Effect of minimum host age on prevalence for pooled *Plasmodium* species (circles), and the age-prevalence pattern expected assuming constant infection rates with age, no infection loss and infection-independent mortality (scenario 1, outlined in Results; triangles). (b) Age-prevalence patterns expected under scenarios 2 (triangles), 3 (diamonds) and 4 (squares), as outlined in Results, shown alongside the observed age-prevalence pattern (circles). Means and 95% confidence intervals are plotted using raw data.

compared to that expected under scenario 1, indicates that at least one of the assumptions is violated ($\chi^2_5 = 25.52$, $P < 0.001$). In subsequent longitudinal analyses, we explored the evidence for two of the above assumptions, specifically whether (i) all infections are maintained and (ii) the risk of infection is age-independent; testing the other assumption(s) is beyond the scope of this paper.

Longitudinal analyses of infection status

The rate of agreement in infection status was high for the same sample tested twice (range 85-97%) and decreased as samples from an individual were taken at longer intervals (Table 2). We found clear evidence that infections could be lost over time: considering pooled *Plasmodium* infections, the probability of infection loss across both years and weeks was significantly higher than that seen when the same sample was tested twice (years: $\chi^2_1 = 14.96$, $P < 0.001$, $n = 128$; weeks: $\chi^2_1 = 4.87$, $P = 0.027$, $n = 102$). However, the patterns of loss appeared to differ for the two species, whereas the proportion of *P. relictum* infections lost across both weeks and years was significantly higher than seen with repeat sample testing (years: $\chi^2_1 = 27.11$, $P < 0.001$, $n = 86$; weeks: $\chi^2_1 = 10.78$, $P = 0.001$, $n = 46$), for *P. circumflexum* there was only evidence for infection loss across years but not weeks (years: $\chi^2_1 = 5.46$, $P = 0.019$, $n = 49$; weeks: $\chi^2_1 = 1.37$, $P = 0.242$, $n = 54$; Fig. 4a). By subtracting the rate of infection loss seen with repeat testing from that observed across years, one can estimate the probability of real (biologically meaningful) infection loss across years. For all *Plasmodium* pooled, this was 13.4%, and was 26.3% for *P. relictum* and 13.8% for *P. circumflexum*.

The probability of infection gain across weeks did not differ significantly from that seen when testing the same sample twice (*P. relictum*: $\chi^2_1 = 0.10$, $P = 0.909$, $n = 173$; *P. circumflexum:* $\chi^2_1 = 0.01$, $P = 0.927$, $n = 181$). However, the rate of infection gain across years was significantly higher than that seen with repeat sample testing, for both *Plasmodium* species (*P. relictum*: $\chi^2_1 = 7.02$, $P = 0.008$, $n = 161$; *P. circumflexum:* $\chi^2_1 = 5.43$, $P = 0.020$, $n = 161$; Fig. 4b). Among individuals that maintained overall *Plasmodium* infection across two time points, there were also cases of species switches. This occurred in 5% of cases when the same sample was tested twice or across weeks (7/136 and 4/75, respectively) and in 13% of cases (11/83) across years. The probability of species switching across years was significantly higher than that for repeat sample testing ($\chi^2_1 = 8.07$, $P = 0.005$) and across weeks ($\chi^2_1 = 7.53$, $P = 0.006$).

We also found evidence that infection risk was age dependent. The risk of infection during the first year of life was taken as the prevalence among yearling birds, whilst the risk of infection between years for 1-, 2- or 3-year-old hosts was determined using longitudinal data from exactly aged birds. We found a marked reduction in infection risk between the first and second years of life, from 0.372 to 0.214 (Fig. 4c; posthoc test of 1st vs. 2nd year infection gain probability, $\chi^2_1 = 10.43$, $P = 0.0012$, all other comparisons $P > 0.20$). When considering *Plasmodium* species separately, infection gain probability differed between the 1st and 2nd year of life for *P. circumflexum* ($\chi^2_1 = 10.56$, $P = 0.001$), whereas this difference was not significant for *P. relictum* ($\chi^2_1 = 1.91$, $P = 0.167$). Equivalent analyses revealed no evidence for age dependency in the probability of infection loss (all comparisons $P > 0.30$).

To test how age dependency of infection and/or loss of infection between years might influence the cross-sectional age-prevalence pattern observed, we calculated the expected age-prevalence pattern under three further scenarios. Scenario 2 included age dependency of infection risk, scenario 3 incorporated a con-

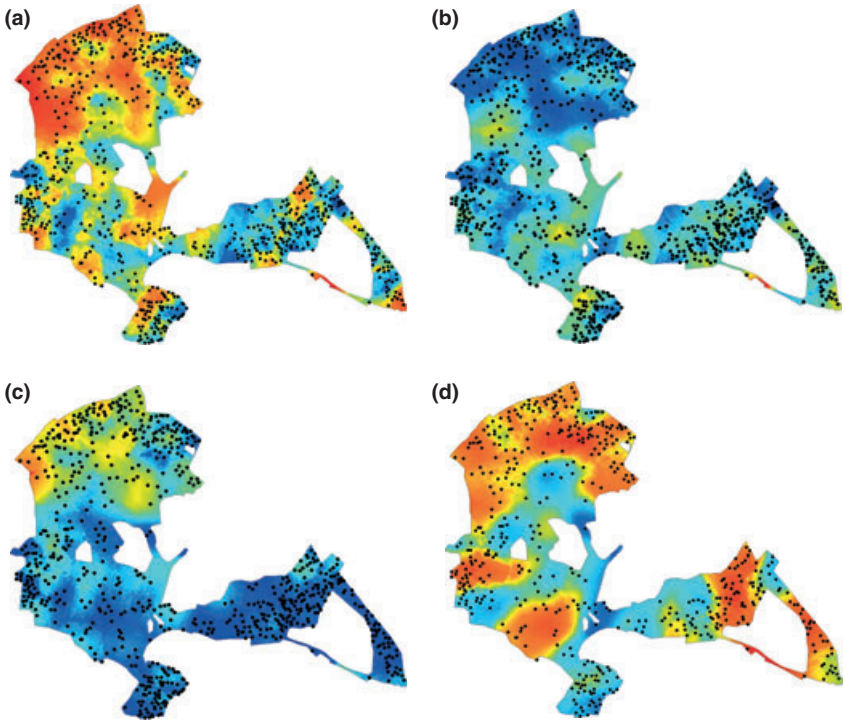

Fig. 3 Interpolations of (a) pooled *Plasmodium* prevalence (b) *P. relictum* prevalence (c) *P. circumflexum* prevalence and (d) parasitaemia among infected individuals (ln(1 + *Plasmodium* DNA copies), as detected by qPCR in the Wytham Woods blue tit population between 2005 and 2008. Areas of high prevalence or parasitaemia appear as red, with orange, yellow, green, pale blue and dark blue indicating areas of decreasing prevalence or parasitaemia.

stant rate of infection loss between years, and scenario 4 incorporated both age-dependent infection risk and infection loss between years. In scenarios incorporating age dependency of infection risk, this was assumed to be 0.372 in the first year of life and 0.214 subsequently, based on longitudinal data. Where applicable, infection loss was included at a rate of 13.4% per year, again based on findings from longitudinal data. Scenarios 2 and 3 both show improved fit to observed data compared to scenario 1, although there is still some evidence for lack of fit under scenario 2 (Fig. 2b; goodness of fit tests: scenario 2 $\chi^2_5 = 10.61$, $P = 0.058$, scenario 3 $\chi^2_5 = 7.93$, $P = 0.160$). Under scenario 4, where both age dependency of infection and infection loss are incorporated, there is no significant discrepancy with observed data (Fig. 2b; $\chi^2_5 = 4.27$, $P = 0.512$).

Cross-sectional analyses of parasitaemia

Mean parasitaemia (among infected individuals only) varied markedly between years (Fig. 5a, Table 3). These temporal fluctuations did not differ between *Plasmodium* species (Table 3) and did not mirror annual changes in prevalence (cf. Figs 1a, b and 5a). For both species, parasitaemia was positively predicted by host age (Fig. 5b, Table 3). Clutch size also predicted para-

Table 2 Proportional rates of agreement in malaria infection status, infection loss and infection gain across three different timescales: when the same sample was tested twice, and when individuals were tested approximately 2 weeks apart or across years

Parasite	Same Sample	2 weeks apart	Across years
All *Plasmodium*			
Agreement rate	0.85	0.81	0.73
Loss	0.16 (0.11, 0.23)	0.25 (0.17, 0.34)	0.30 (0.23, 0.38)
Gain	0.14 (0.10, 0.20)	0.15 (0.10, 0.22)	0.24 (0.18, 0.31)
P. relictum			
Agreement	0.87	0.82	0.73
Loss	0.24 (0.18, 0.30)	0.44 (0.32, 0.58)	0.50 (0.40, 0.60)
Gain	0.09 (0.07, 0.12)	0.09 (0.06, 0.15)	0.16 (0.11, 0.22)
P. circumflexum			
Agreement	0.94	0.95	0.88
Loss	0.17 (0.12, 0.24)	0.11 (0.05, 0.23)	0.31 (0.20, 0.45)
Gain	0.04 (0.03, 0.06)	0.04 (0.02, 0.08)	0.08 (0.05, 0.13)

Agreement rate is given by the proportion of diagnoses that were identical. Rates of loss and gain are given with 95% confidence intervals.

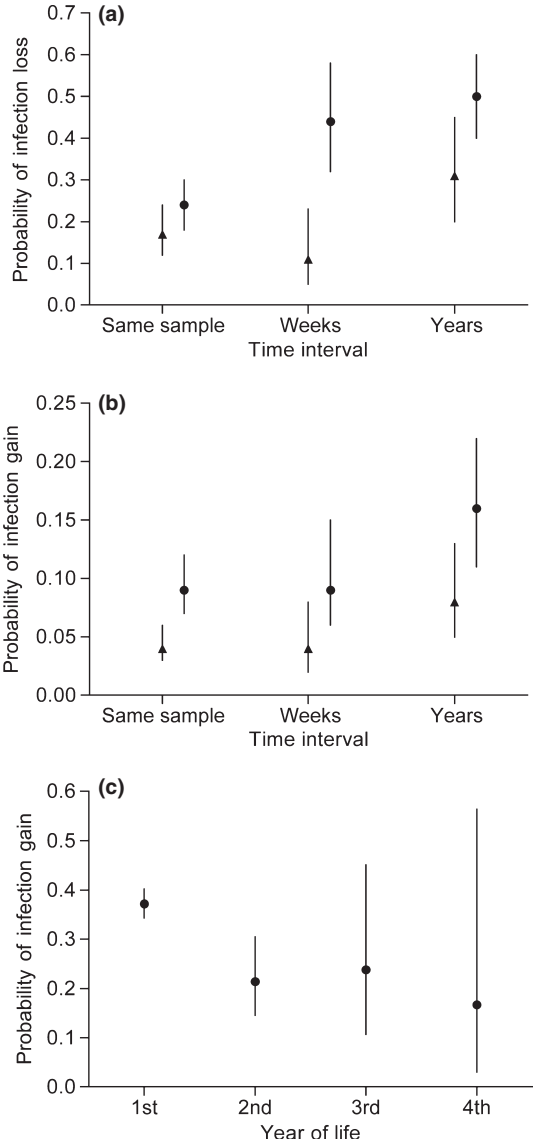

Fig. 4 Probability of *Plasmodium* infection (a) loss and (b) gain across different temporal timescales: when the same blood sample was tested twice, or when birds were tested across either an approximately 2-week period or across years. Mean probabilities are plotted with 95% confidence intervals for *Plasmodium relictum* (circles) and *P. circumflexum* (triangles). Symbols for the two species are offset for clarity. (c) Probability of infection gain according to host age.

sitaemia, such that birds with a larger clutch size tended to have higher infection intensity (Fig. 5c; Table 3). A significant effect of nestbox y coordinate indicated the presence of broad-scale spatial variation in parasitaemia, with infection intensity tending to be higher in the north of the woodland (Table 3). However, an interpolated map of parasitaemia indicated a heterogeneous distribution of heavily infected birds, rather than any consistent spatial trend (Fig. 3d). Spatial variation in parasitaemia did not obviously relate to spatial variation in prevalence, as mean parasitaemia did not predict prevalence across arbitrarily defined 0.25 km^2 grid squares ($F_{1,24} = 0.26$, $P = 0.613$, $r = 0.104$; analysis weighted by N individuals in each grid square, including only grids with ≥10 individuals). However, several local environmental characteristics predicted parasitaemia, including the number of oaks near a nestbox (Oaks50), as well as the interaction between EDI and altitude; birds breeding in oak-rich territories had lower parasitaemia (Fig. 5d, Table 4), and the interaction between EDI and altitude indicated that whereas near the woodland edge altitude had little effect on parasitaemia (which may be because there is little altitudinal variation here), in the centre of the woodland birds breeding at higher altitude had lower parasitaemia (Table 4). All interactions between these environmental variables and *Plasmodium* species were non-significant (Oaks50*Species $F_{1,601} = 0.63$, $P = 0.427$, EDI*Species $F_{1,601} = 1.02$, $P = 0.312$, Altitude*Species $F_{1,601} = 1.77$, $P = 0.184$, EDI*Altitude*Species $F_{1,600} = 0.00$, $P = 0.969$). Because tits at the edge of this woodland are more likely to be immigrants (Wilkin *et al.* 2007a), we tested whether immigrant status (whether a bird was born and ringed in a local nestbox) could explain the edge effect detected. However, immigrant status did not predict parasitaemia ($F_{1,588} = 1.84$, $P = 0.176$) and its inclusion did not influence the edge effect.

Longitudinal analyses of parasitaemia

Repeatability of parasitaemia decreased as the time interval between tests increased, with $r = 0.83$ for the same DNA extraction tested twice, $r = 0.51$ among infected samples from females taken approximately 2 weeks apart, and $r = 0.39$ for samples taken from the same individual across one or more years. Among individuals infected in multiple years, we performed a variance component analysis to examine the relative importance of individual identity and year in explaining parasitaemia variation. Significance for both random effects was assessed using log-likelihood ratio tests, that is, by testing the change in deviance when each random effect was excluded from the full model against the chi-squared distribution. Whereas year explained a relatively small proportion of the variance in parasitaemia (2.9%; $\chi^2_1 = 3.73$, $P = 0.053$), individual identity explained over a third of the variance in this trait (38.3%; $\chi^2_1 = 21.59$, $P < 0.001$).

To assess whether within-individual increases in parasitaemia could underlie the cross-sectional pattern of increasing parasitaemia across age groups, we tested the effect of minimum host age in a mixed model containing individual identity as a random effect, including

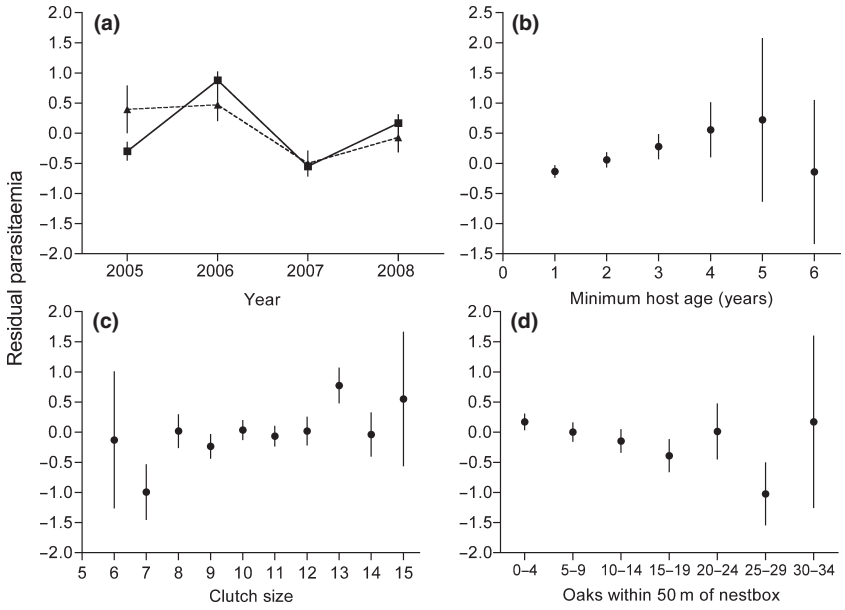

Fig. 5 Predictors of parasitaemia among *Plasmodium*-infected blue tits at Wytham Woods: (a) Year (b) Minimum host age (c) Clutch size and (d) Oak richness of breeding location, measured as the number of oaks within a 50 m radius of the nestbox. In (a), the within-individual pattern of annual changes in parasitaemia (triangles) is shown alongside the cross-sectional pattern (squares). In all plots, means and standard errors are plotted and data are residuals from the appropriate minimal model excluding only the factor of interest (see Tables 3 and 4 for details of covariates). Data for longitudinal patterns in (a) were derived from a model including minimum host age as a fixed effect and individual identity as a random effect, amongst all individuals infected on more than one occasion.

data from individuals with parasitaemia estimates from at least two years (n = 191 samples from 90 individuals). Minimum age was significant in this model ($F_{1,121}$ = 4.21, P = 0.043), suggesting parasitaemia increases within individuals as they age. The magnitude of this within-individual age effect was similar to that seen for host age in cross-sectional analysis (r = 0.132 vs. r = 0.101 respectively). Although it is theoretically possible that such an effect could arise from age-related selective appearance or disappearance of individuals with high or low parasitaemia (van de Pol & Verhulst 2006), we found no convincing evidence this was the case (see Data S1 in Supporting information). This model also retained a significant effect of year ($F_{3,150}$ = 4.03, P = 0.009) indicating that parasitaemia fluctuated within individuals across years, in a pattern very similar to annual differences in parasitaemia observed in cross-sectional analyses (Fig. 5a).

Discussion

Analysis of individual variation in avian malaria infection status and parasitaemia within a single wild bird population revealed a complex set of environmental and host influences underlying these traits. Longitudinal analyses further showed that these infections, although thought to be chronic and long lasting, show marked temporal dynamics within naturally infected wild hosts.

Spatiotemporal variation in prevalence and parasitaemia

Annual changes in prevalence were pronounced in this population, and were mirrored by those occurring in yearlings, suggesting that annual differences in parasite transmission drive these effects. However, both temporal as well as spatial prevalence patterns differed notably for two common species of *Plasmodium*, with *P. relictum* showing marked annual fluctuations and a southerly distribution, whilst *P. circumflexum* exhibited more stable prevalence across years and a northerly distribution (Figs 1a, b and 3). Parasitaemia also showed marked interannual and spatial variation within the population, yet these patterns did not obviously parallel those seen for prevalence (cf. Figs 1a, b, 5a and 3a, d). Thus, although it has been shown previously (Bentz *et al*. 2006), and in the present study, that parasitaemia affects the probability of detecting *Plasmodium* infections, this effect appears not to be sufficient to drive patterns of prevalence in this population, at least when employing the relatively sensitive diagnostic method of qPCR. Indeed, the differences in temporal and spatial patterns of prevalence and parasitaemia found here,

Table 3 Factors predicting parasitaemia among *Plasmodium*-infected individuals in the population (n = 640)

Predictor	df	F	P	r
Minimum age	1	6.14	**0.014**	0.098
Minimum age^2	1	0.02	0.901	−0.006
Year	3	12.31	**<0.001**	
Clutch size	1	7.63	**0.006**	**0.109**
HD_{std}	1	0.54	0.464	−0.029
Sex (female)	1	0.17	0.680	−0.016
Species (*P. relictum*)	1	0.17	0.684	0.016
x	1	2.83	0.093	0.067
y	1	5.10	**0.024**	**0.089**
x*y	1	0.00	0.954	0.002
Minimum age*Species	1	0.00	0.951	0.003
Minimum age^2 *Species	1	0.82	0.366	0.037
Year*Species	3	1.84	0.139	
HD_{std}*Species	1	0.37	0.543	−0.024
Sex*Species	1	0.67	0.413	−0.033
x*Species	1	0.16	0.686	−0.016
Clutch size*Species	1	0.70	0.402	−0.034
y*Species	1	1.81	0.179	−0.054
x*y*Species	1	1.38	0.240	0.047

All variables in the starting model are listed, with effects in the minimal model in bold. For significant effects (P < 0.05), statistics are from the minimal model; for non-significant effects, statistics are presented from the last model that included this term. Effect sizes (Pearson's r) are given for all effects with a single degree of freedom.
HD_{std}: standardized hatch date, calculated as described in methods; x and y are the geographical coordinates of an individual's nestbox.

Table 4 Local environmental variables predicting parasitaemia among *Plasmodium*-infected blue tits in Wytham Woods (n = 637)

Predictor	df	F	P	r
Year	3	12.95	**<0.001**	
Minimum age	1	6.36	**0.012**	**0.100**
Clutch size	1	7.61	**0.006**	**0.109**
EDI	1	5.97	**0.015**	**0.097**
Altitude	1	5.07	**0.025**	**−0.089**
EDI*Altitude	1	4.18	**0.041**	**−0.081**
Oaks50	1	5.33	**0.021**	**−0.092**
Territory size	1	0.17	0.684	−0.017

All variables in the starting model are listed, with effects in the minimal model in bold. For significant effects (P < 0.05), statistics are from the minimal model; for non-significant effects, statistics are presented from the last model that included this term. Effect sizes (Pearson's r) are given for all effects with a single degree of freedom.

combined with differences in other predictors found for parasitaemia but not prevalence (Tables 1 and 3), suggest quite different biological processes underpin variation in these two traits. The spatial prevalence patterns found here mirror those from a previous study on this population (Wood *et al.* 2007), which showed that for *P. circumflexum* and pooled *Plasmodium* species, prevalence was highest in the northwest of Wytham, closer to the adjacent River Thames (Fig. 3a, c). Recent findings suggest mosquito abundance is highest in this area (R. Alves *et al.* unpublished data), and it seems likely that variable exposure to infective vectors, for example determined by the abundance of preferred larval breeding habitats plays a key role in driving this fine-scale spatial variation in malaria prevalence. If *P. relictum* and *P. circumflexum* are transmitted by vector species that differ in habitat requirements (a possibility supported by laboratory-based vector competency studies of these species; Valkiūnas 2005), this could explain their contrasting spatiotemporal patterns. However, spatial variation in prevalence may not only reflect environmental variation in transmission potential, but also potentially host-driven processes such as non-random dispersal (e.g. Garant *et al.* 2005) with respect to infection status, a possibility that warrants further investigation.

Whereas spatiotemporal prevalence patterns clearly differed between *Plasmodium* species, the factors predicting parasitaemia showed no species-specificity. Thus, whereas parasite-specific processes, such as particulars of transmission biology, appear to influence whether a host becomes infected, individual parasitaemia may depend more on host characteristics such as immunological status or genetic resistance, or environmental changes that affect all hosts (and therefore probably both *Plasmodium* species) approximately equally. Investigations of *Plasmodium* parasitaemia in other populations are needed to assess the generality of this finding.

Several descriptors of the local breeding environment predicted parasitaemia in this population, including the oak richness of an individual's territory, with birds on oak rich territories tending to have lower parasitaemia. Oak richness can be considered a measure of territory quality, as it affects nestling diet quality and fledging mass among tits in Wytham (Wilkin 2006; Wilkin *et al.* 2009). Several processes could conceivably underlie a negative correlation between oak richness and parasitaemia, including direct effects of diet quality on immune function (Lochmiller *et al.* 1993), or improved immune function as a result of reduced energetic expenditure required to provision young in high quality habitats (Stauss *et al.* 2005). Consistent with the latter possibility, we found that parents making a larger reproductive effort (those with larger clutch sizes) had higher *Plasmodium* parasitaemia (Fig. 5c). Furthermore, there is consistent published evidence for causal effects of reproductive effort on blood parasitaemia and immune function (Knowles *et al.* 2009). Although higher intrinsic quality of birds inhabiting oak-rich territories (Przybylo

et al. 2001) could alternatively explain the negative association between oak richness and parasitaemia, several results argue against this: high quality individuals may be expected to breed relatively early, in larger territories and produce large clutch sizes, yet none of these factors negatively predicted parasitaemia in this study (see Tables 3 and 4), and in fact the association with clutch size is positive.

Within-individual parasitaemia variation

Parasitaemia showed significant repeatability within infected hosts, and individual identity explained nearly 40% of the variance in this trait among infected individuals between years. There is therefore clear potential for genetic or permanent environmental effects on this trait. However, parasitaemia was also labile within individuals with respect to host age, as well as fluctuating within individuals across years (Fig. 5a). Thus, it seems parasitaemia is sensitive to changes in either host physiological state and/or environmental conditions, though these effects are subtler than the influence of individual identity. The finding that parasitaemia shows parallel annual dynamics within individuals is intriguing and suggests that annually varying environmental factors influence parasitaemia similarly in all individuals and that there may be 'good' and 'bad' years in terms of parasitaemia. One possibility is that immune suppression of malaria infection competes with other demands on hosts that vary between years, such as the need for thermoregulation or foraging effort.

Plasmodium parasitaemia increased across host age cohorts (Fig. 5b) and longitudinal analyses indicated that this effect was largely due to within-individual increases in parasitaemia as they age. These findings are contrary to documented patterns for *Haemoproteus* infections, in which parasitaemia has been shown to decline as hosts get older (Sol *et al.* 2003; Stjernman 2004). Declining parasitaemia among *Plasmodium*-infected humans is also the norm (e.g. Syafruddin *et al.* 2009). Thus, the age-related increase in parasitaemia reported here, although statistically well supported, is unexpected. It may be that declines in parasitaemia reflecting acquired immunity would be observed on a shorter timescale than across years, or only among individuals that are able to control or eventually clear infections, which appears to be possible (at least for *P. relictum,* see below) on much shorter timescales. The pattern of increasing parasitaemia with age cannot therefore be interpreted as a lack of effective acquired immunity against these parasites. Indeed, clear evidence for development of immunity against avian *Plasmodium* parasites exists in the experimental literature (Atkinson *et al.* 2001; Graczyk *et al.* 1994). One possibility is that an age-related increase in parasitaemia reflects a process of immunosenescence among individuals that maintain chronic infections. Age-related declines in several measures of cell-mediated and humoral immune responsiveness have been documented in birds (Lavoie 2005; Palacios *et al.* 2007).

Within-individual changes in infection status

Through longitudinal analysis, malaria infection status within individuals was shown to be non-static, with infections disappearing from the blood across both short (2 week) and long (across years) timescales. Whilst accounting for detection failure, up to 26% of patent *P. relictum* infections were lost on a fortnightly or between-year basis. If these effects were due simply to parasitaemia fluctuating such that infections sometimes drop below our assay's detection limit, we would expect infections to appear across the same timescales they disappear. However, this was not observed (cf. Fig. 4a, b for *P. relictum* across weeks), suggesting the process of infection disappearance we observe is one-way. Several processes could explain the apparent loss of infections, including (i) clearance of infection, i.e. sterilizing immunity (ii) persistence of blood-stage parasites below the assay detection threshold or (iii) parasite sequestration within fixed tissues, which is known to occur for both *P. relictum* and *P. circumflexum* in experimental infections (Valkiūnas 2005) and is strongly suggested by seasonal prevalence patterns in wild populations (Cosgrove *et al.* 2008). Long-term monitoring of experimental *Plasmodium* infections in canaries has shown that although parasitaemia often becomes microscopically undetectable for weeks or months, parasites are still present, as naïve hosts inoculated with blood from such individuals often develop infections (Manwell 1934). Regardless of how infections disappear from the blood, a consequence is that a significant undetectable reservoir of exposed and potentially infective individuals may be missed when infections are diagnosed solely using DNA- or microscopy-based detection of blood-stage parasites. The dynamic nature of infection status within individuals, coupled with the pronounced temporal and fine-scale spatial effects on prevalence reported here, suggest single time-point assessments of infection status may provide a poor indicator of host genetic resistance to avian malaria in wild birds. Measuring and accounting for variation in exposure will therefore be critical when attempting to measure resistance in natural settings. One promising approach would be to measure antibody levels (Atkinson *et al.* 2001; Jarvi *et al.* 2002) as well as parasitaemia, and thus permit a comparison of infection intensity among individuals known to have been exposed.

Although prevalence increased across age cohorts in this population (Table 1, Fig. 2a), the risk of becoming infected declined with host age: whilst infection risk in the first year of life was nearly 40%, infection risk in subsequent years was approximately halved (Fig. 4c). This reduced infection risk among older hosts could have a number of explanations. One possibility is that some individuals are resistant to infection and thus never develop chronic infections. A recent study has demonstrated that for *P. relictum* (cyt *b* lineage pSGS1), hosts show marked inter- and intraspecific variation in resistance to experimental infection (Palinauskas *et al.* 2008). The recovery of several Hawaiian honeycreeper species from the threat of extinction and re-establishment in areas of high malaria prevalence also highlights the potential for intraspecific variation in resistance to avian malaria (Foster *et al.* 2007). Consistent individual differences in behaviour, such that some individuals persistently avoid infection whilst others are quickly infected, could also play a role in this pattern. The potential for such processes in a population like that studied here is considerable, given that there is strong spatial variation in the risk of infection and that blue tits show high breeding site fidelity after postnatal dispersal, with around 25% breeding in the same nestbox in multiple years and over 90% in the same broad sector of the woodland (B.C. Sheldon, unpublished data). The stronger age-dependency in infection risk for *P. circumflexum* compared to *P. relictum* found here is consistent with such a behavioural explanation, as the spatial distribution of *P. circumflexum* prevalence is far more repeatable across years compared to *P. relictum* (S. Knowles, unpublished data). Experimental infection studies on a wider range of host species are needed to provide further insight into the extent of intraspecific variation in resistance to avian malaria. When combined, the two processes of infection loss and age-dependency of infection risk were able to account for the observed quadratic relationship between host age and prevalence found in cross-sectional analysis. This suggests that additional effects of infection-related mortality or dispersal are not necessary to explain this age-prevalence pattern, although dedicated survival analysis would be required to test definitively for such processes.

Conclusion

The findings here illustrate the ecological complexity of avian malaria epidemiology that can be observed within a single host population. Strong spatiotemporal effects as well as host factors influenced within-population heterogeneity in both infection status and parasitaemia. Thus, both these infection traits are underpinned by a combination of environmental and host factors, and simplistic assumptions about prevalence reflecting either exposure or intrinsic resistance among individuals or populations, are unrealistic. Moreover, infections within individuals were clearly dynamic, with a significant proportion of patent infections (over 20% for one *Plasmodium* species) disappearing from the blood on both short and long timescales, despite the use of sensitive qPCR diagnostics. These dynamics imply that population surveys using single time-point assessments of infection may often fail to recognize a reservoir of exposed, and potentially infective, individuals. The ability to distinguish between unexposed and exposed but immune individuals is likely to be critical for future studies of these parasites in wild populations, and efforts to further develop methods for achieving this should be a priority.

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Supporting information

Additional Supporting information may be found in the online version of this article.

Table S1 Comparison of *Plasmodium* detection by the nested PCR method of Waldenström *et al.* (2004), and a novel quantitative PCR assay, among samples tested by both methods

Table S2 Results from GLMM modelling of *Plasmodium* infection status (prevalence) variation across the Wytham blue tit population

Fig. S1 Increase in cumulative *Plasmodium* prevalence as more qPCR tests are performed. Means and 95% confidence intervals are shown.

Data S1 Supplementary information.

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Fitness effects of endemic malaria infections in a wild bird population: the importance of ecological structure

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Summary

1. Parasites can have important effects on host populations influencing either fecundity or mortality, but understanding the magnitude of these effects in endemic host–parasite systems is challenging and requires an understanding of ecological processes affecting both host and parasite.
2. Avian blood parasites (*Haemoproteus* and *Plasmodium*) have been much studied, but the effects of these parasites on hosts in areas where they are endemic remains poorly known.
3. We used a multistate modelling framework to explore the effects of chronic infection with *Plasmodium* on survival and recapture probability in a large data set of breeding blue tits, involving 3424 individuals and 3118 infection diagnoses over nine years.
4. We reveal strong associations between chronic malaria infection and both recapture and survival, effects that are dependent on the clade of parasite, on host traits and on the local risk of infection.
5. Infection with *Plasmodium relictum* was associated with reduced recapture probability and increased survival, compared to *P. circumflexum*, suggesting that these parasites have differing virulence and cause different types of selection on this host.
6. Our results suggest a large potential survival cost of acute infections revealed by modelling host survival as a function of the local risk of infection.
7. Our analyses suggest not only that endemic avian malaria may have multiple fitness effects on their hosts and that these effects are species dependent, but also that adding ecological structure (in this case parasite species and spatial variation in disease occurrence) to analyses of host–parasite interactions is an important step in understanding the ecology and evolution of these systems.

Key-words: avian malaria; plasmodium, blue tits (Cyanistes caeruleus), host–parasite interactions, life-history trade-offs, multistate mark–recapture models

Introduction

Parasites that reduce the fitness of their hosts will constitute a strong selective force in natural populations and have the potential to exert important evolutionary and ecological pressures in the wild (Poulin 2007). It is now widely acknowledged that the negative impacts of parasites on host fitness can play an important role in regulating host populations (Albon *et al*. 2002), in driving host population cycles (Hudson, Dobson & Newborn 1998) and, in the extreme, can even limit population persistence and viability (McCallum *et al*. 2009; Smith, Acevedo-Whitehouse & Pedersen 2009). In addition to potential effects on population dynamics, the manner in which parasites reduce host fitness can also influence host life-history evolution (Sheldon & Verhulst 1996), especially because host immune defences and behavioural traits may evolve in different ways in response to impacts on fecundity as opposed to survival, or to impacts incurred at different life-history stages (Lochmiller & Deerenberg 2000; Moller, Martin-Vivaldi & Soler 2004). Hence, to understand the ecological and evolutionary implications of parasites on their hosts, it is essential to quantify host–parasite interactions, particularly the effects of parasites on hosts in the wild.

Malaria parasites (e.g. *Haemoproteus*, *Plasmodium*) and their avian hosts have frequently been used as a model system to investigate host–parasite interactions, co-evolutionary processes and the role of parasites in host life-history evolution (Galvani 2003; Ricklefs, Fallon & Bermingham 2004; Ricklefs & Outlaw 2010). Nevertheless, despite much research, the impacts of blood parasites on host fitness and

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host population dynamics in the wild remain poorly understood. In naive host populations, malaria parasites have sometimes been shown to dramatically increase host mortality, reduce population abundance and limit species distributions (van Riper *et al*. 1986; Atkinson & van Riper 1991; Atkinson & Samuel 2010). They have also been shown to have detrimental impacts on host survival in captive and domestic birds (Atkinson & van Riper 1991; Williams 2005; Palinauskas *et al*. 2008). However, in wild populations where malaria transmission is endemic and hosts have a long evolutionary history with these parasites, studies provide conflicting results as to whether such infections have appreciable fitness effects for hosts (e.g. Korpimäki, Hakkarainen & Bennett 1993; Sanz *et al*. 2001; Marzal *et al*. 2005; Bensch *et al*. 2007).

There are several reasons why detecting the impacts of malaria parasites on host fitness in endemic areas is difficult. As the acute stage of malaria infection (when parasitaemia is high) is very brief and can entail significant mortality costs, the vast majority of infected individuals in natural populations will be 'survivors', which as a result of host-acquired immunity harbour only chronic infections (Valkiūnas 2005; Atkinson & Samuel 2010). Hosts with chronic infections show greatly reduced parasitaemia levels and may bear minimal fitness costs of infection (Valkiūnas 2005; Bensch *et al*. 2007). Moreover, if the impacts of acute infections occur over short time scales and the impacts of chronic infections are small, then in the absence of direct experimentation (e.g. Knowles, Palinauskas & Sheldon 2010a) detecting effects on hosts will require both large sample sizes and long-term data on the traits of infected and uninfected individuals in endemic populations (McCallum & Dobson 1995). Such longitudinal studies of the infection dynamics of avian malaria in wild populations where transmission is endemic remain rare.

The assessment of fitness costs of malaria infection in the wild is also complicated by the considerable diversity of malaria species that may comprise infections in host populations (Bensch *et al*. 2004; Waldenström *et al*. 2004). Pathogen virulence may vary among species, and parasite species may also differ in the nature of their impacts on hosts (Lively 2006; Palinauskas *et al*. 2008). Hence, the presence of multiple, cryptic or unrecognized malaria species within a host population can potentially obscure any fitness effects. To date, few studies have explicitly considered the possibility that infection dynamics and host–parasite interactions may vary with malaria species (Wood *et al*. 2007; Marzal *et al*. 2008; Ortego *et al*. 2008).

Another important, but often overlooked, problem when attempting to assess disease impacts in the wild is the issue of detectability or capture heterogeneity (Jennelle *et al*. 2007). Pathogen-induced changes in behavioural traits, activity levels or other physiological processes can lead to significant heterogeneity in the probability of sampling infected and uninfected individuals (Senar & Conroy 2004). If disease-dependent variation in capture probabilities exists but is ignored, then observed patterns (e.g. prevalence or survival rates) and any inference based on them may simply be artefacts of host encounter rates or conceal important biologically relevant effects because of biases (Jennelle *et al*. 2007). The majority of ecological studies of host–parasite interactions in avian malaria systems have not considered issues of state-dependent detectability. This is particularly worrying in populations infected with multiple malaria species, as it is possible that hosts infected with different species are encountered at different rates, particularly if there is spatial variation in the distribution of those species (e.g. Wood *et al*. 2007). Multistate mark–recapture models (MSMR) provide a framework for assessing disease impacts in wild populations, while explicitly accounting for variability in detection rates with infection status (Conn & Cooch 2009). Although fast becoming an integral tool in wildlife disease ecology, such models have rarely been used to assess fitness consequences in avian malaria systems (Vanderwerf 2008; Atkinson & Samuel 2010).

In this study, we utilized MSMR models to assess the fitness consequences of malaria in a long-term monitored population of blue tits (*Cyanistes caeruleus*), infected with two divergent *Plasmodium* parasite species (*P. relictum* and *P. circumflexum*, Valkiūnas 2005). We aimed to determine whether these malaria parasites have significant fitness effects for hosts when transmission is endemic and in particular the extent to which impacts of infection differ with respect to the *Plasmodium* species infecting individuals and as a function of host traits (age and sex).

Materials and methods

STUDY SITE AND HOST SPECIES

Blue tits are small passerine birds that take readily to nestboxes. In the UK, blue tits are resident year-round and lay eggs in spring with the peak of broods hatching (in southern England) from late April to early May. From 2001 to 2009, 250–450 pairs of individually marked blue tits were monitored in Wytham Woods (51°46′N, 1°20′W), near Oxford, UK. The 385-ha study site is a continuous mixed semi-deciduous forest (complete description of study area in Perrins 1979), in which approximately 1160 nestboxes are distributed at variable densities. Blood samples for infection diagnosis were collected annually from breeding blue tits captured between day 6 and 14 of the nestling phase, either within the nestbox by hand or using traps, or with mist nets in front of the nest entrance. As the study population is single brooded and breeding is highly synchronous, there is little variation in the calendar date among samples within each year (average range ± SE = 42·42 ± 6·78 days). However, the proportion of all captured birds from which blood samples were obtained (and analysed) did vary across years (Table 1). Host sex was determined based on the presence (female) or absence (male) of a brood patch, while age (yearling or adult) was determined using plumage characteristics (Svensson 1992) or ringing records for birds ringed as nestlings. As an exact age could not be assigned for a significant proportion (23%) of captured adults, we restricted age effects in survival analyses to two age classes: yearlings (1 year olds) and adults (2+ year olds).

AVIAN MALARIA PARASITES AND MOLECULAR DIAGNOSIS OF INFECTION

Previous molecular characterization of haemosporidian infections in this population has shown that infections of two well-defined

Appendix 2

Table 1. Total number of individuals (and of each sex) captured in each year of the study and the number of these for which diagnoses were and were not obtained (by disease state and by parasite species)

Year	No. captured (No. F/M)	No. tested for malaria	Molecular method	No. State unknown*	No. species unknown†	Apparent prevalence for species combined (±95% CI)	Apparent/corrected prevalence‡ *Plasmodium relictum*	*P. circumflexum*
2001	429 (230/199)	182	Nested PCR	247	1	0·151 (0·110, 0·224)	0·066/0·088	0·088/0·069
2002	517 (283/234)	0	NA	517	NA	NA	NA	NA
2003	572 (318/254)	48	Nested PCR	524	0	0·101 (0·034, 0·227)	0·063/0·082	0·042/0·032
2004	473 (244/229)	398	Nested PCR	75	9	0·302 (0·257, 0·349)	0·138/0·187	0·138/0·109
2005	494 (273/221)	472	Nested PCR & qPCR	66	32	0·521 (0·475, 0·567)	0·248/0·339	0·214/0·171
2006	475 (273/202)	472	Nested PCR & qPCR	3	17	0·445 (0·399, 0·491)	0·218/0·287	0·210/0·161
2007	523 (304/219)	517	Nested PCR & qPCR	6	25	0·484 (0·440, 0·528)	0·310/0·386	0·149/0·109
2008	525 (284/241)	523	qPCR	2	7	0·363 (0·322, 0·406)	0·136/0·184	0·214/0·169
2009	509 (259/250)	506	qPCR	3	7	0·389 (0·347, 0·433)	0·121/0·167	0·251/0·203

Also shown are apparent prevalence estimates (number infected/total individuals captured) for malaria species combined and both apparent and corrected prevalence estimates for each species separately.

*State was unknown for individuals that were not tested for *Plasmodium* infections.

†Species was unknown for individuals not tested for *Plasmodium* infection, for mixed infections (infections with both *Plasmodium* Species), and for infections in which the parasite species could not be ascertained.

‡Prevalence estimates were corrected for state-dependent recapture rates using mean state-dependent recapture rates from model p(St + boxD) (see Table 4) according to the following formula:

$$\mathrm{Prev}_{\mathrm{corr}} = \frac{\frac{C_{\mathrm{cladeA}}}{p_{\mathrm{cladeA}}}}{\left(\frac{C_{\mathrm{cladeA}}}{p_{\mathrm{cladeA}}} + \frac{C_{\mathrm{cladeB}}}{p_{\mathrm{cladeB}}} + \frac{C_{\mathrm{Uninf}}}{p_{\mathrm{uninf}}}\right)}$$

where C is the count of individuals in different states and p is the recapture rate for that state.

Plasmodium morphospecies (based on cytochrome *b* sequences), *P. relictum* and *P. circumflexum* (Palinauskas *et al.* 2007) are common during the breeding season and constitute >98% of blood parasites in the genera *Plasmodium* and *Haemoproteus* (Knowles *et al.* 2011). We refer to these parasites by their morphospecies classification, but to simplify model notation (see CMR results below) we refer to *P. relictum* as 'R-Clade' and *P. circumflexum as* 'C-Clade'. Although 'morphospecies' classifications can conceal much greater species diversity at a finer level of molecular resolution (Bensch *et al.* 2004), in this population both clades consisted of predominantly one parasite lineage (cytochrome *b* sequence, see Table S1). Moreover, phylogenetic analyses revealed far greater divergence between the two clades than among any of the lineages within the clades (Wood *et al.* 2007). For these reasons, and also because the multistate models are very data intensive, we restrict our comparisons to these two historically divergent morphospecies ('species' for simplicity).

Samples were screened for parasites either by nested polymerase chain reaction (PCR) assays from 2001 to 2004 (protocols described in Wood *et al.* 2007; Waldenström *et al.* 2004 and) or quantitative (q)PCR assays from 2005 to 2009 (protocols details described in Knowles *et al.* 2011). DNA quantification during qPCR assays revealed that, as expected, the majority of infected hosts harboured chronic infections with very low parasitaemia loads (>90% of infected hosts possessed parasitaemia values two orders of magnitude lower than the maximum values recorded, unpub. data).

A proportion of samples were screened by both methods (30%, Table 1) and revealed a high degree of concordance both in the diagnoses given by the two methods (78%; $n = 1042$) and in the malaria species assigned to infected diagnoses (96%, $n = 302$; Knowles *et al.* 2011). However, because qPCR assays involved analyses of samples in triplicate (with a positive diagnosis if at least one replicate was positive), whereas nested PCR assays involved a single analysis of each sample, the overall detection rate for qPCR was greater than for nested PCR (82% of mismatches involved a positive diagnosis by qPCR and a negative diagnosis by nested PCR). Where diagnoses differed, individuals were considered uninfected only if both methods gave uninfected diagnoses. If either method gave an infected diagnosis, then individuals were considered to be infected. Hence, we assume that false positives by either method are negligible. This was justified because strict laboratory protocols minimized false positives: results were only used when all negative controls on a PCR plate showed no contamination; samples that returned equivocal results were retested and if still ambiguous then they were designated as 'unknown' in model analyses.

Although false-positive diagnoses will be rare in this study, false-negative diagnoses may have occurred. If not accounted for, false-negative diagnoses will introduce negative bias in survival estimates. A recent simulation study has revealed, however, that when the probability of false positives is low and the true detection probability is at least 50%, then provided at least three samples are tested per unit there will be very little bias in estimates (McClintock *et al.* 2010). Because the majority of diagnoses in this study were undertaken with qPCR in which samples are analysed in triplicate, and because only two survival estimates were obtained in the years in which nested PCR was used (see Results), we believe that the potential for biased survival estimates to produce spurious inferences in this study is small. Moreover, exclusion of years 2001–2003 from analyses did not qualitatively change the results of recapture and survival rate modelling presented below.

Mixed species infections were rare in this population (<5% of all infections) and were treated as 'infected' in the combined-species analysis and as 'unknown' in the species-specific analysis, as were the few occasions ($N = 46$) where there was disagreement between the two molecular methods in the *Plasmodium* species diagnosed (see Knowles *et al.* 2011 for species diagnosis methods).

MSMR MODELLING APPROACH

The mark–recapture data set consisted of yearly capture histories for all breeding birds captured within the study site from 2001 to 2009,

grouped by sex and age and assigned to different disease states according to their infection status at the time of capture. Because not all individuals were tested for malaria in all years, the infection status of a proportion of birds was not known (Table 1). To accommodate these undiagnosed individuals within our MSMR framework, we employed newly developed 'multievent' models, which explicitly account for unknown or partially observable states by treating them as a hidden Markov process (Pradel 2005; Conn & Cooch 2009). We employ the general model structure described in Conn & Cooch (2009), which allows for both the detection process and the process of obtaining data on infection status conditional on being detected to be modelled (see Fig. 2 in Conn & Cooch 2009). Incorporating unknown disease states directly into the estimation process increases the precision of parameter estimates and is a significant improvement over the alternative options of either censoring such individuals or assigning them to a separate state (Faustino *et al.* 2004; Conn & Cooch 2009).

We conducted two multievent mark–recapture analyses to assess the impacts of endemic malaria in our study population and demonstrate the importance of accounting for parasite species. In the first analysis, we combined both *Plasmodium* species into a single infected state to examine the overall impact of endemic malaria infection on host survival and recapture. The data set for this analysis included all captured individuals, regardless of whether information on their infection status was known. Capture histories were assigned to one of three events (captured and infected, captured and uninfected, captured but infection status unknown) corresponding to two disease states (infected and uninfected, see Fig 1a; the 'dead' state is explicitly included in all multievent models, Pradel 2005). In the second analysis, we kept both *Plasmodium* species separate and examined species-specific impacts. In this analysis, capture histories were assigned to one of four events (captured with R-Clade infection, captured with C-Clade infection, captured and uninfected, captured but infection status unknown), corresponding to three disease states (uninfected, infected with R-Clade, infected with C-Clade, see Fig 1b). In both analyses, transitions between all states were possible. Here, we report the results of survival and recapture rate modelling; a further paper (Lachish *et al. submitted*) will report the results of transition rate modelling and assess patterns of infection and recovery rates within the population.

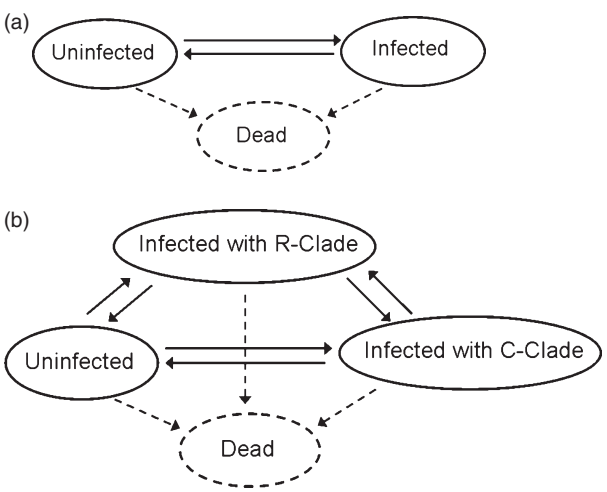

Fig. 1. Multievent mark–recapture model structure for (a) combined *Plasmodium* analysis and (b) species-specific analysis (C-Clade = *P. circumflexum*; R-Clade = *P. relictum*) used for modelling survival and recapture rates of blue tits.

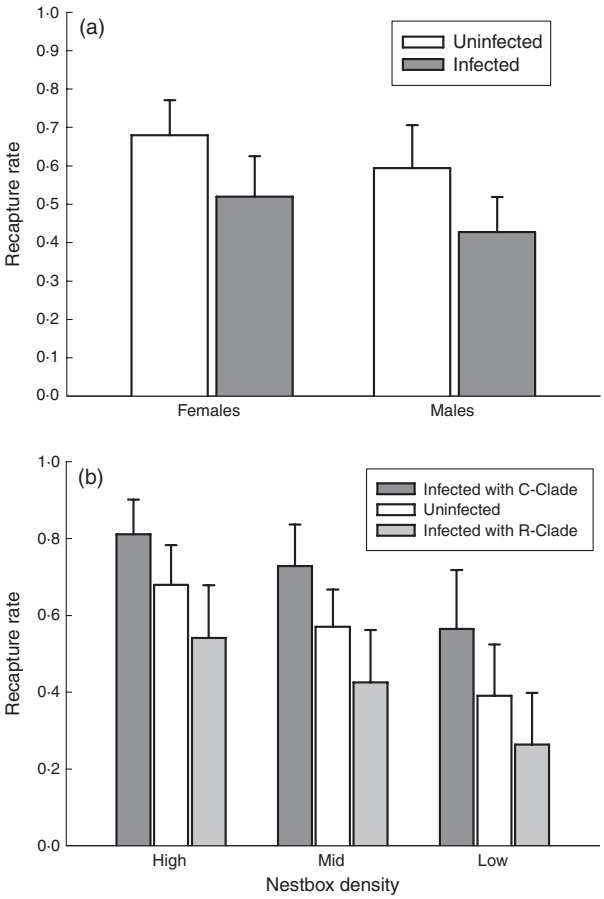

Fig. 2. Results of multievent mark–recapture modelling of recapture rates showing (a) the effect of combined *Plasmodium* infections on recapture rates of uninfected and infected, male and female blue tits; and (b) effect of *P. relictum* (R-Clade) or *P. circumflexum* (C-Clade) infection on the recapture rate of blue tits in areas of high, medium or low nestbox density. Estimates are model averaged means ± 95% CI.

We aimed to quantify differences in recapture and survival rates among infected and uninfected individuals and assess whether effects of infection differed in relation to host factors. Our global models thus included the effects of disease state, time (yearly variation), host sex and host age. Unfortunately, our data were too sparse to fit full time-dependent models for all parameters. In both analyses, the initial capture probability (which estimates the probability of being in a given state when first encountered, Pradel 2005) was time invariant and set to vary with disease state. Also in both analyses, we set the partial observation parameter (which estimates the underlying probability of observing disease state, given that the individual is captured, Conn & Cooch 2009) to vary only by disease state (infected vs. uninfected) and the molecular protocol used for diagnosis (nested PCR vs. qPCR). In addition, in the species-specific analysis, changes in infection status from R-Clade to C-Clade infection or vice versa were modelled as time invariant, as these transitions were extremely sparse in our data set. As goodness-of-fit tests are not currently available for reduced parameter multievent models (Pradel 2005; Choquet *et al.* 2009), we accounted for potential lack of fit in our data in the following ways: (i) by including models with trap dependence in capture probabilities in the candidate model set to account for the possibility of heterogeneity in recapture rates between individuals; (ii) by including models with age effects to account for the possibility of

transience (an excess of newly marked individuals that are never seen again), and (iii) by using a reasonably large variance inflation factor ($\hat{c}$ = 1·5) for conservative model selection (Faustino *et al*. 2004; Choquet *et al*. 2009).

For each analysis, we first modelled variation in recapture rates in relation to disease state, time, trap dependence and sex (preliminary results yielded no evidence that recapture rates varied with age), with survival and transition rates fully parameterized. To assess whether observed differences in clade-specific recapture rates (see Results) were driven by factors that were spatially confounded with the distribution of the two *Plasmodium* species in the population, rather than infection with the parasite per se, we also included models in which recapture rate varied with the density of nestboxes in the woodland area in which individuals were captured or with the distance individuals were from the edge of the woodland (to allow for greater emigration by individuals in the edge). Each of the nine woodland areas in the study site (see Fig. 2 in Wood *et al*. 2007) was classified as having either high (> 7 nestboxes/ha: C, B), medium (3–4 nestboxes/ha: O, P, MP, CP, W) or low (< 2 nestboxes/ha: E, SW) nestbox density. Distance from the edge of the study site was calculated per nestbox using GIS, with each nestbox classified as being either near (within 100 m) or far from the woodland edge.

Next, we modelled variation in survival rates in relation to disease state, time (year), sex and age, using the most parsimonious recapture rate model identified in step one. In addition to models with state-dependent survival rates, we also included models in which host survival rates varied between areas of high and low *Plasmodium* prevalence (preliminary investigations showed this measure of infection risk to be much better supported than measures involving only one or other of the *Plasmodium* species). These models were included to explore the possibility of acute effects of infection on host survival. We reasoned that if purportedly uninfected hosts have lower survival rates in high-prevalence areas, where the force of infection is high, then this would indicate that acute infections carry a fitness cost for hosts (because birds in high-prevalence areas would be more likely to have acquired infection and died soon after infection without this transition appearing in our data set). Average interpolated *Plasmodium* prevalence from 2005 to 2009 (the years when qPCR was used) was calculated per nestbox using inverse distance weighted interpolation (described in Wood *et al*. 2007). We chose 40% prevalence (close to the mean prevalence; Table 1) as a cut-off point to distinguish high-prevalence from low-prevalence sites.

In mark–recapture analyses, survival is confounded with permanent emigration. We assessed whether the observed differences in the apparent survival rates of C-Clade and R-Clade infected individuals (see Results) could be attributed to differences in emigration rates in two ways. First, we included models in the candidate set in which survival rates varied between individuals captured close to and far from the woodland edge, to verify that edge-dwelling individuals were not more likely to 'emigrate' (less likely to survive). Second, we compared the distance moved within the study site (breeding dispersal distances) of uninfected individuals and individuals infected with the two clades as an index of likely emigration rate (assuming that greater within-site movement might predict an increased propensity to emigrate). We also assessed whether the observed survival differences were because of underlying habitat quality by comparing the abundance of oak trees within 50 m of nestboxes (a strong predictor of habitat quality and breeding success, Perrins 1991) in high and low-prevalence areas (Wilkin, Perrins & Sheldon 2007, Wood *et al*. 2007).

All models were fitted to the data using program E-SURGE (Choquet 2009). To ensure convergence of models on the global minima, models were run using repeated random initial values ('multiple random' option with N = 8; Choquet 2007). We investigated the additive and interactive effects of model variables up to two-way interactions between main effects (more complicated models were not well supported). Model selection was based on small sample size corrected Akaike Information Criteria adjusted for overdispersion (QAICc), with models that differed in QAICc values by < 2 considered equivalent in their ability to describe the data (Burnham & Anderson 2002). The relative likelihood of each model in a candidate set was estimated with normalized QAICc *w*eights (w_i, or the index of relative plausibility). We obtained robust parameter estimates through model averaging (Burnham & Anderson 2002) and, where cited, effect sizes of disease state on recapture and apparent survival rates (on the logit scale) were also model averaged from relevant models. Model notation is explained in Table 2.

Table 2. Notation used to denote the main effects and model structure for modelling recapture rate (p) and apparent survival rate (Φ)

Model notation	Description	Parameter
St	State-dependent effect	p, Φ
U	Uninfected individuals	p, Φ
I	Infected individuals (clades combined)	p, Φ
R	Individuals infected with R-Clade (*Plasmodium relictum*)	p, Φ
C	Individuals infected with C-Clade (*P. circumflexum*)	p, Φ
Covariate effects		
Sx	Sex effect	p, Φ
a2	Age effect (yearlings or adults)	Φ
boxD	Density of nestboxes (low/mid/high)	p
edge	Distance from the edge of the woodland (near/far)	p, Φ
prev	Local prevalence of *Plasmodium* (high/low)	Φ
trap	Trap dependence effect	p
t	Time dependence (yearly variation)	p, Φ

Results

A total of 3424 birds were captured an average of 1·4 times for a total of 4843 captures over the nine years of the study (Table 1). We observed 175 transitions between known disease states (the uninfected and infected states) in the combined-species analysis and 167 transitions among known disease states (the uninfected and the two infected states) in the species-specific analysis (all transitions including those involving unknown disease states were more numerous: 381 for the combined analysis and 354 for the clade-specific analysis). In years where > 80% of individuals were tested, the apparent prevalence of *Plasmodium* in the population ranged from 30 to 52%, with the prevalence of *P. relictum* (R-Clade) more variable among years than the prevalence of *P. circumflexum* (C-Clade; Table 1).

RECAPTURE RATES

All the most parsimonious models in the combined-species analysis contained an effect of disease state on recapture rates

Table 3. Summary results of the multievent mark–recapture analysis modelling the effect of combined *Plasmodium* infections on recapture and survival rates of blue tits

Parameter	Model	k	Deviance	QAICc	ΔQAICc	*w*
(a) Recapture rates (p)	St + Sx†	47	11170·91	7533·30	0	0·394
	St	46	11175·71	7535·28	1·982	0·146
	St × Sx	48	11169·69	7535·85	2·546	0·110
	St + trap	47	11172·84	7535·90	2·601	0·107
	St × trap	48	11172·81	7537·42	4·123	0·050
	t + Sx	53	11158·21	7537·90	4·602	0·039
	t	52	11163·76	7539·56	6·256	0·017
	St + t + Sx	54	11158·19	7539·93	6·633	0·014
	Trap	42	11183·04	7540·17	6·869	0·013
	St + t	49	11163·65	7541·53	8·232	0·006
(b) Survival rates (Φ)	(U × prev) + I + a2 + Sx	34	11195·53	7524·10	0	0·232
	a2 + Sx	32	11204·02	7525·71	1·611	0·120
	t + a2 + Sx	39	11182·89	7525·83	1·725	0·113
	prev + t + a2 + Sx	40	11180·13	7526·02	1·915	0·103
	a2	31	11208·75	7526·84	2·736	0·068
	t + a2	38	11188·33	7527·42	3·315	0·051
	a2 × Sx	33	11204·00	7527·72	3·621	0·044
	St + a2 + Sx + t	40	11182·81	7527·80	3·701	0·042
	t + a2 × Sx	40	11182·87	7527·84	3·739	0·041
	(St + prev) + a2 + Sx	41	11180·10	7528·04	3·939	0·037

The top ten models in each candidate set are shown. k = number of parameters; *w* = model weight. † Most parsimonious recapture rate model retained for modelling survival rates. See Table 2 for model notation.

(Table 3a); with estimates showing that uninfected birds had higher recapture rates than infected birds (Fig. 2a). However, there was also very strong support for a disease effect in recapture rates in the species-specific analysis, indicating that recapture rates varied not only between uninfected and infected birds, but also between birds infected with different malaria species (Table 4a). Although the top model in the species-specific analysis allowed recapture rates to vary between all three states, two pieces of evidence indicate that the strong support for a disease effect was entirely because of differences between the different malaria species. First, models in which the recapture rates of the uninfected state

Table 4. Summary results of the multievent mark–recapture analysis modelling the effect of *Plasmodium relictum* ('R' Clade) and *P. circumflexum* ('C' Clade) infections on recapture and survival rates of blue tits

Parameter	Model	*k*	Deviance	QAICc	ΔQAICc	w_i
(a) Recapture rates (p)	St + boxD*	74	10499·93	7134·63	0	0·374
	St + boxD with St = UR v C	73	10503·78	7135·11	0·481	0·294
	St + boxD with St = UC v R	73	10505·54	7136·28	1·657	0·163
	St + boxD + Sx	75	10499·36	7136·72	2·098	0·131
	boxD	72	10514·86	7140·42	5·789	0·021
	St + boxD with St = U v RC	73	10514·10	7141·99	7·361	0·009
	St × boxD	78	10507·12	7147·75	13·123	0·001
	St	72	10531·53	7151·53	16·904	0·000
	St + trap	73	10530·31	7152·79	18·168	0·000
	St + Sx	73	10531·39	7153·52	18·893	0·000
	St + edge	74	10530·35	7154·90	20·277	0·000
(b) Survival rates (Φ)	(St + t) + (R/C × a2) + (U × prev)	50	10553·35	7137·10	0	0·434
	(St + t) + a2 + (U × prev)	58	10554·90	7138·13	1·032	0·259
	(St + t) + a2	57	10562·61	7141·21	4·108	0·056
	(St + t) + a2 + prev	60	10553·68	7141·44	4·343	0·049
	(St + t) + (R/C × a2)	58	10559·91	7141·48	4·373	0·049
	(St + t) + a2 *with St = UR v C*	56	10567·63	7142·50	5·399	0·029
	(St + t) + (St × a2)	59	10558·52	7142·61	5·504	0·028
	(St + t) + a2 + Sx	58	10562·54	7143·23	5·865	0·023
	(*St + t*) + *a2 with St = UC vs. R*	56	10569·10	7143·48	6·126	0·020
	(St + t) + (St × a2) + Sx	56	10558·41	7144·599	7·497	0·010

The top ten models in each candidate set are shown. *k*, number of parameters; *w*, model weight.
*The most parsimonious recapture rate model retained for modelling survival rates. See Table 2 for model notation.

were combined with those of either R-Clade or C-Clade states received equivalent support to the top model, whereas a model in which R-Clade and C-Clade states were combined received substantially less support (Table 4a). Second, the 95% confidence interval for the effect of C-Clade state relative to R-Clade state on recapture rates did not include zero [average effect size on the logit scale (±95% CI) = 1·03 (0·49, 1·42)], whereas those for either of the infected states relative to the uninfected state did [R-Clade relative to the uninfected state = −0·29 (−0·27, 0·21), and C-Clade relative to the uninfected state = 0·41 (−0·01, 0·81)].

Recapture rates for individuals infected with R-Clade malaria were lower than those of individuals infected with C-Clade malaria, while the recapture rates of uninfected individuals were intermediate to the two infected states (Fig. 2b). The variation in recapture rates between malaria species could not be attributed to the proximity of individuals to the woodland edge, nor to spatial variation in nestbox density (Table 4a). However, there was strong support for an additive effect of nestbox density on recapture rates (Table 4a). In this population, blue tits were more likely to be captured in areas of greater nestbox density (Fig. 2b).

There was some support for an additive effect of sex in recapture rates in the combined-species analysis (Table 3a) with estimates showing that females were more likely to be captured than males (Fig. 2a). However, in the species-specific analysis sex differences in recapture rates were no longer strongly supported (Table 4a). In both analyses, models with yearly variation in recapture rates were not well supported, indicating that encounter rates were relatively constant throughout the study. There was also little support for trap dependence in recapture rates in either analysis (Tables 3a and 4a).

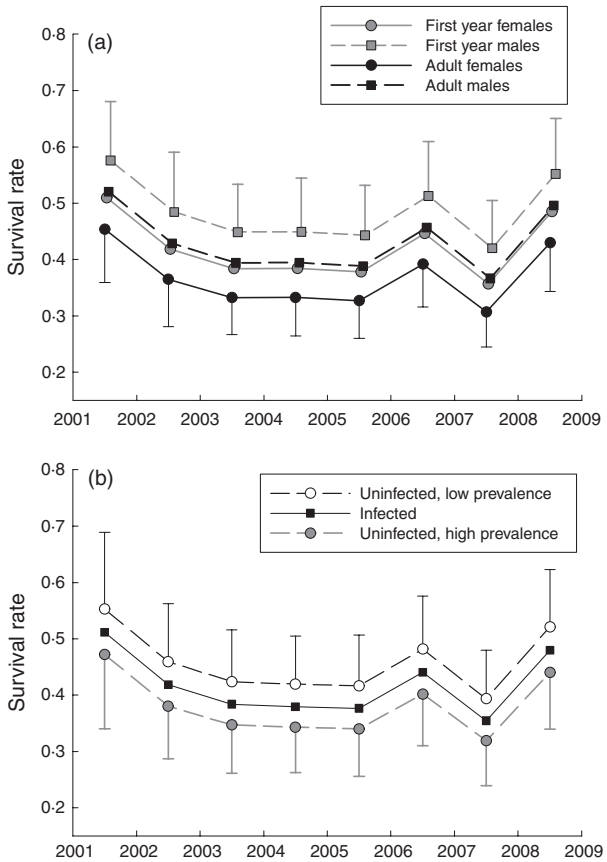

Fig. 3. Results of combined *Plasmodium* multievent mark–recapture modelling of survival rates showing (a) survival rates of blue tits as a function of host age and sex and (b) the effect of disease risk (population prevalence) on survival of blue tits. Estimates are model averaged means ± 95% CI. To clarify patterns, not all error bars are shown.

APPARENT SURVIVAL RATES

In the combined-species analysis, the most parsimonious model in the candidate set included only the effects of age and sex on host survival, with equivalent support for models with either constant or yearly variation in survival rates (Table 3b). Adult survival rates were on average lower than those of yearlings, while female survival rates were lower than those of males (Fig. 3a). In this analysis, there was little support for models in which survival rates varied between disease states (Table 3b), suggesting that malaria infections do not impact greatly on host survival. However, as discussed earlier, we are likely to have missed most acute infections in this population. If acute infections carry fitness costs, then survival rates should be lower in areas where there is a higher force of infection (and thus greater risk of infection). Results showed strong support for models in which survival rates of individuals varied between areas of high and low malaria prevalence, with more weight for the model in which only the survival rate of uninfected individuals varies with disease prevalence (Table 3b). This result thus suggests that acute malaria infections do cause significant mortality for hosts in this population. Estimates from this model show that survival rates of uninfected individuals were on average 17% lower in high than in low-prevalence areas (Fig. 3b). This result did not appear to be driven by an underlying correlation between high habitat quality and low *Plasmodium* prevalence as low-prevalence sites contained far fewer Oak trees than did high-prevalence areas ($t = -3{\cdot}155$, df = 799, $P < 0{\cdot}001$). A consequence of missed infections (those occurring outside of our sampling periods) is that parameter estimates for the uninfected state will be biased, because some individuals classified as uninfected will have become infected between sampling occasions. This bias likely explains why recapture and survival rates for uninfected individuals were found to be intermediate to those of individuals infected with the two *Plasmodium* species.

In the species-specific analysis, as a result of the paucity of information on infection status in the early years of this study (no malaria testing in 2002 and species diagnoses for only five individuals captured in 2003), survival rates could not be estimated in 2003 [confidence intervals for these estimates were either abnormally large (from 0 to 1) or small (equal to zero)]. In contrast to the combined-species analysis, there was strong support for time variation in survival rates in this analysis

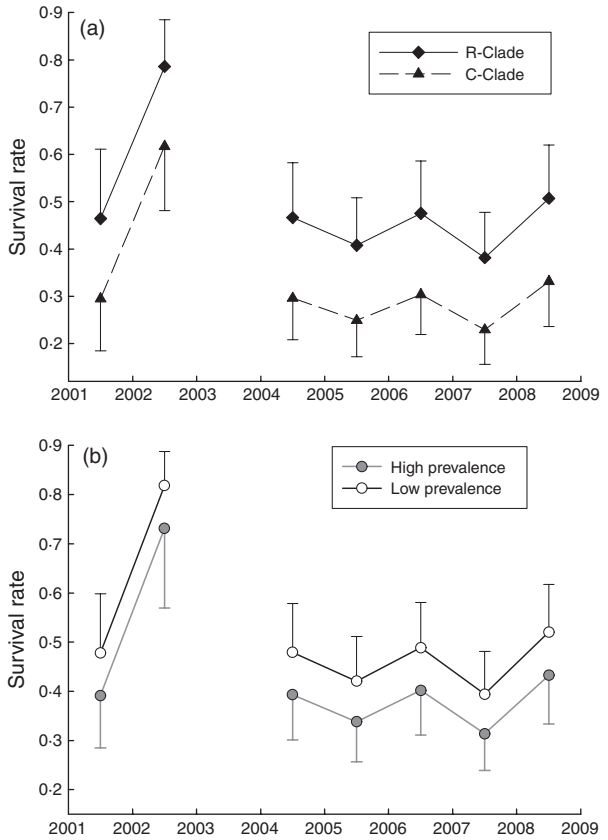

Fig. 4. Results of species-specific multievent mark–recapture analysis of survival rates showing (a) effect of *Plasmodium relictum* (R-Clade) or *P. circumflexum* (C-Clade) infection on the survival rates of blue tits; and (b) effect of disease risk (population prevalence) on survival rates of uninfected blue tits. Estimates are model averaged means ± 95% CI.

that appeared to be driven by a single particularly high survival rate of individuals from 2002 to 2003 (Table 4b, Fig. 4). Also in contrast to findings with *Plasmodium* species combined (Table 3b), results of the species-specific analysis revealed very strong support for a disease effect on survival rates (all the top models in the candidate set included an effect of disease state on survival rate; denoted 'St' in Table 4b), indicating that the two malaria species differed in their impacts on host survival. Models in which the survival rates of all three states differed received more support (ΔQAICc > 5) than models in which any of the states were combined (denoted 'UR vs. C' or 'UC vs. R' in Table 4b). However, as reported for recapture rates above, the only significant effect of disease state on survival rates was that of C-Clade relative to R-Clade state (average effect size on the logit scale (±95% CI) = −0·708 (−1·46, −0·35). Survival rates of individuals infected with C-Clade malaria (*P. circumflexum*) were on average 31% lower than the survival rates of individuals infected with R-Clade malaria (*P. relictum*) (Fig. 4a). Again, as detailed earlier, models in which the survival rate of uninfected individuals varied between high and low-prevalence areas were also strongly supported here (Table 4b), with the reduction in the survival rates of uninfected individuals in high-prevalence areas similar to that in the species-combined analysis (Fig. 4b).

In contrast to the combined-species analysis, the best-supported models in the species-specific analysis included only an effect of host age and not sex on survival rates (Table 4b). Overall adult survival rates were 14% lower than the survival rates of yearling birds, which was similar to the difference observed in the combined-species analysis. There was also some support for models in which this age effect was restricted to infected states only (equivalent support for models '(St + t) + a2' and '(St + t) + (R/C × a2)'; Table 4b), suggesting differential impacts of malaria infection on adults and yearlings. Model selection revealed little support for models in which survival rates varied with the distance individuals were from the woodland edge. There was also no difference in the average within-site movement of uninfected individuals and individuals infected with different malaria species ($F_{(2,793)}$ = 1·170, P = 0·3118).

Discussion

Understanding the ecological and evolutionary implications of parasites requires knowledge of their effects on hosts in natural populations. Avian blood parasites have long served as a model system for investigating host–parasite interactions yet their impacts on host fitness in endemic conditions remain very poorly understood. Difficulties in detecting the effects of endemic malaria infections can be attributed to infections being comprised of multiple cryptic species, to the fact that the majority of individuals in natural populations harbour only chronic infections and to sampling inequalities between disease states. In this study, by incorporating information on parasite diversity and spatial variation in disease risk, and by accounting for state-dependent detectability in a mark–recapture framework, we were able to demonstrate effects suggesting that both acute and chronic malaria infections entail substantial fitness costs for hosts in a population where transmission is endemic. More importantly, our results revealed that the two malaria species within our population impacted in contrasting ways on host fitness components and will thus impose very different selective pressures on hosts in this population.

The costs of acute *Plasmodium* infections can be severe for naive hosts in regions where avian malaria has been recently introduced (Atkinson & Samuel 2010) but have never been quantified in natural populations where hosts have co-evolved with these parasites. In this study, blue tits were sampled for infection status annually, at a fixed point in their annual cycle, such that many within-year infections were presumably missed, particularly as peak *Plasmodium* transmission may be outside our sampling period (Cosgrove *et al.* 2008). Thus, like many studies investigating host–parasite interactions in avian malaria systems (Bensch *et al.* 2007; Marzal *et al.* 2008), we could not directly measure the acute effects of malaria infection on hosts. Nonetheless, by explicitly incorporating information on the spatial variability of the force of infection within our population, we were able to

demonstrate that survival rates of individuals, particularly uninfected individuals, in high-prevalence areas were considerably lower than those of individuals in low-prevalence areas. This result did not appear to be driven by correlated differences in habitat quality (at least in terms of the abundance of oak trees). Moreover, that uninfected individuals suffered a greater survival cost than infected individuals in high-infection-risk areas argues against this pattern being a result of other spatially correlated confounding factors or differential emigration rates (see also the discussion below). We suggest this result provides evidence of an acute effect of *Plasmodium* infection. However, we cannot discount the possibility that this result might also reflect differential settlement patterns of high- and low-quality individuals (individuals with intrinsically higher survival rates prefer low-prevalence areas). Confirmation that acute *Plasmodium* infections entail appreciable mortality costs for hosts in this wild population will ultimately require a much finer temporal sampling scale (days to weeks): a logistically difficult task to implement.

Acknowledging the potential for *Plasmodium* species to differ in their effects on hosts greatly improved our ability to detect impacts of chronic infections on host fitness in this species. With the addition of species-specific information, model selection revealed that survival rates of individuals infected with *P. circumflexum* were substantially lower than individuals infected with *P. relictum*; explaining the absence of a disease state effect on survival rates in the combined-species analysis. That the combined effects of multiple malaria species may mask the underlying impacts of any one species could be responsible for the failure of many studies to detect fitness effects of chronic malaria infections in the wild (e.g Bensch *et al.* 2007; Ortego *et al.* 2008; Marzal *et al.* 2008). Indeed, this is the first study in a wildlife species to demonstrate differences in survival rates between individuals of a single host species infected with different malaria species (though such effects are apparent in humans, Clark & Schofield 2000).

There is little reason to suspect that these observed differences in apparent survival rates were an artefact of differential emigration rates: survival rates did not vary with the location of individuals within study site, as might be expected if edge-dwelling individuals were more likely to emigrate. Further, within-site dispersal distance (an index for emigration propensity) did not differ between uninfected individuals and individuals infected with either *P. relictum* or *P. circumflexum*. Instead, the considerable difference in survival rates between individuals infected with *P. relictum* and *P. circumflexum* suggests that *P. circumflexum* is more virulent than *P. relictum*. Although it is not entirely unexpected that the virulence of different malaria species may vary within a single host species, few studies to date have examined such effects (Atkinson & van Riper 1991), particularly in wild populations. Although the generality of the pattern observed here remains to be seen, an evolved lower virulence in *P. relictum* might explain the much broader documented host range and larger global distribution of this species compared with *P. circumflexum* (Bensch, Hellgren & Perez-Tris 2009; Hellgren, Perez-Tris & Bensch 2009).

Alternatively, infections with different *Plasmodium* species may induce varied behavioural or physiological changes in hosts that could either increase the exposure of hosts to other sources of mortality (e.g. predation, Moller & Nielsen 2007) or predispose hosts to secondary infections (Poulin 1994). Indeed, if individuals infected with *P. circumflexum* suffer multiple parasite infections, this may explain the observed higher virulence of *P. circumflexum* malaria relative to *P. relictum*, as within-host competition between parasites is assumed to select for higher virulence (Choisy & de Roode 2010). The possibility that hosts infected with *P. circumflexum* experience lower survival rates as a result of either greater predation pressures or greater parasite burdens has important implications for our understanding of the way these parasites can shape host populations and warrants further investigation.

Another plausible explanation for differences in survival rates between individuals infected with different malaria species is if infection triggers a change in reproductive output that results in increased survival, owing to reduced costs of reproduction (Sheldon & Verhulst 1996). In this study, recapture rates of individuals infected with *P. relictum* were significantly lower than for individuals infected with *P. circumflexum*. As only birds that were actively breeding were captured in this study, one explanation of this pattern is that infection with *P. relictum* causes early breeding failure in hosts. Support for this suggestion is provided by two recent studies. The first, in a neighbouring population of blue tits to that studied here, found that infections with *Plasmodium* parasites (predominantly *P. relictum* in this population) were associated with nest abandonment prior to hatching (Knowles, Palinauskas & Sheldon 2010a). Second, as shown by Knowles, Wood & Sheldon (2010b), infection by *P. relictum* exacerbates the cost of increased brood size in terms of reproductive output in blue tits. Hence, as has been shown for other avian blood parasites in other hosts (Marzal *et al.* 2005; Ortego *et al.* 2008), it appears that chronic *P. relictum* infections entail a number of significant reproductive costs for breeding blue tits. Individuals infected with *P. relictum* were observed to have higher survival rates than individuals infected with *P. circumflexum*, suggesting that the diversion of energy away from reproduction might benefit their long-term survival. A similar pattern has been observed for rodents infected with cowpox virus, whereby higher survival rates of infected hosts relative to uninfected hosts were attributed to the fact that infected rodents expend less reproductive effort because infection caused individuals to delay reproduction until the following breeding season (Telfer *et al.* 2002, 2005).

Our results showing that recapture and survival rates differed in opposing ways in individuals infected with the two malaria species raise the possibility that blue tits modify their reproductive strategies in a manner consistent with the theoretical predictions regarding adaptive host life-history responses to parasitism (Perrin, Christe & Richner 1996;

Agnew, Koella & Michalakis 2000). Individuals infected with *P. relictum,* which may have evolved lower pathogenicity, are able to invest to a greater degree in immune resistance (as infection compromises their current reproduction effort) and thus experience higher survival rates. Conversely, as *P. circumflexum* appears more virulent, individuals may respond to infection by maximize their current reproductive effort when survival prospects are challenged. Experimental work is clearly needed to conclusively evaluate the role of these malaria species in the resource allocation strategies employed by blue tits.

A key finding from this study was that recapture rates differed substantially between uninfected and infected individuals and between individuals infected with different *Plasmodium* species. We also found clear evidence that capture probabilities of birds varied independently as a function of nestbox density. These effects were very well supported and large: the probability of capturing a bird infected with *P. circumflexum* in an area with high density of nestboxes was 68% greater than for a bird infected with *P. relictum* in an area of low nestbox density (Fig. 2b). Such variation will be a very important source of bias in prevalence estimates and estimates of demographic parameters in studies that have not used mark–recapture methods to account for differences in detectability. For example, correcting species-specific prevalence estimates for state-dependent recapture rates in our population results in prevalence estimates that are on average 33% higher for *P. relictum* and 22% lower for *P. circumflexum* (see Table 1). Such capture heterogeneity among individuals infected with different malaria species will have profound implications for studies in which 'apparent' prevalence is used to compare the effects of different malaria species, (Crespin *et al*. 2008).

This study is one of the few to have demonstrated that malaria infections have significant negative consequences for host fitness in a wild population where transmission is endemic. More importantly, this study revealed that different malaria species can have very different effects on host fitness components in a single host species. Crucially, these results were only apparent with the inclusion of information on the diversity of *Plasmodium* infections within our population, as well as information on the spatial variation in risk of infection within the study site. This highlights the importance of considering genetic variability among parasites and the ecological context of the host–parasite interaction when studying the consequences of endemic infections on natural populations. The magnitude of the impacts of chronic *P. circumflexum* infections on survival rates, the strong indications of acute effects of *Plasmodium* infection, together with the inferred impact of *P. relictum* infections on host reproduction clearly indicate that malaria infections should no longer be dismissed as having negligible effects on their host in endemic regions (Ricklefs & Outlaw 2010). Indeed, that these two malaria species differ so greatly in their effects on host fitness indicates they will likely impose very different selective pressures on hosts in this population. Investigating how such differential selection pressures contribute to the evolution of genetic aspects of host resistance and host life-history strategies is a fruitful area for further research.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Table S1. Diversity of avian malaria lineages in the Wytham Woods blue tit population.

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Appendix 3

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Infection dynamics of endemic malaria in a wild bird population: parasite species-dependent drivers of spatial and temporal variation in transmission rates

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Summary

1. Investigating the ecological context in which host–parasite interactions occur and the roles of biotic and abiotic factors in forcing infection dynamics is essential to understanding disease transmission, spread and maintenance.

2. Despite their prominence as model host–pathogen systems, the relative influence of environmental heterogeneity and host characteristics in influencing the infection dynamics of avian blood parasites has rarely been assessed in the wild, particularly at a within-population scale.

3. We used a novel multievent modelling framework (an extension of multistate mark–recapture modelling) that allows for uncertainty in disease state, to estimate transmission parameters and assess variation in the infection dynamics of avian malaria in a large, longitudinally sampled data set of breeding blue tits infected with two divergent species of *Plasmodium* parasites.

4. We found striking temporal and spatial heterogeneity in the disease incidence rate and the likelihood of recovery within this single population and demonstrate marked differences in the relative influence of environmental and host factors in forcing the infection dynamics of the two *Plasmodium* species.

5. Proximity to a permanent water source greatly influenced the transmission rates of *P. circumflexum*, but not of *P. relictum*, suggesting that these parasites are transmitted by different vectors.

6. Host characteristics (age/sex) were found to influence infection rates but not recovery rates, and their influence on infection rates was also dependent on parasite species: *P. relictum* infection rates varied with host age, whilst *P. circumflexum* infection rates varied with host sex.

7. Our analyses reveal that transmission of endemic avian malaria is a result of complex interactions between biotic and abiotic components that can operate on small spatial scales and demonstrate that knowledge of the drivers of spatial and temporal heterogeneity in disease transmission will be crucial for developing accurate epidemiological models and a thorough understanding of the evolutionary implications of pathogens.

Key-words: Avian malaria, blue tits, *Cyanistes caeruleus*, disease incidence rate, environmental heterogeneity, host age, host sex, multievent mark–recapture models, *Plasmodium*, transition rates

Introduction

Transmission is a key epidemiological process for understanding host–pathogen dynamics and predicting the effects of disease on populations (McCallum, Barlow & Hone 2001). In addition to biotic factors, such as host age, sex or abundance, parasite transmission in natural populations may be influenced by abiotic factors, such as microclimate and landscape (Hudson *et al.* 2002). For example, transmission of vector-borne pathogens will be at least partly governed by environmental traits that limit vector abundance and the spatial and temporal distribution of vectors (Sinski *et al.* 2006; Byers *et al.* 2008). Because transmission processes can be proximately driven by local conditions, spatial heterogeneity

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in environmental factors will play an important role in mediating infection dynamics and the spread and persistence of disease (Byers *et al*. 2008; Osnas *et al*. 2009).

In addition to effects on transmission, environmental heterogeneity may also alter various components of host fitness. For example, host condition, nutritional status or stress level may vary with habitat quality resulting in differential impacts of pathogens over relatively small spatial scales (Lafferty & Kuris 1999; Beldomenico *et al*. 2009). Hence, the environment in which hosts and parasites interact can also substantially affect the strength and specificity of selection (Wolinska & King 2009). Ultimately, spatial variability in the drivers of host infection and in pathogen-induced selection pressure on hosts will direct patterns of local adaptation and co-evolutionary processes (Kaltz & Shykoff 1998; Dybdahl & Storfer 2003). Thus, investigating the ecological context in which host–parasite interactions occur and the roles of biotic and abiotic factors in forcing infection dynamics is essential to understanding the dynamics of disease spread and maintenance in the wild and the evolutionary implications of parasites for hosts. Such knowledge is particularly relevant in a world facing significant climate and anthropogenic change but has received scant attention for most wildlife diseases (Daszak, Cunningham & Hyatt 2001; Harvell *et al*. 2009).

Avian malaria (*Plasmodium* or *Haemoproteus* spp. Valkiūnas 2005) are globally distributed vector-borne parasites commonly used as model systems for testing hypotheses in evolutionary ecology (Ricklefs, Fallon & Bermingham 2004; Knowles, Nakagawa & Sheldon 2009), and investigating diagnostic traits and control options for human malaria (Slater 2005). Studies of human malaria (*P. falciparum*) have revealed transmission rates to be largely governed by environmental factors, such as altitude and proximity to water, which restrict the distribution of mosquito vectors (Foley *et al*. 2003; Balls *et al*. 2004). It is likely, however, that host demographic factors could play a more significant role in the transmission of avian relative to human malaria because synchronized breeding seasons generate periodic recruitment of immunologically naive juveniles to host populations and because disparity in reproductive behaviours of male and female birds may differentially affect their exposure to vectors (Arriero & Moller 2008; Cosgrove *et al*. 2008). Although correlations between host prevalence and environmental variables have been documented for avian malaria species at a range of spatial scales (Atkinson *et al*. 2005; Freed *et al*. 2005; Wood *et al*. 2007; O'Connor, Dudaniec & Kleindorfer 2010), variation in population prevalence may not necessarily reflect variation in the underlying transmission rates (Anderson & May 1979; Bolzoni, Real & De Leo 2007). Moreover, prevalence estimates may be subject to significant bias if the detection probabilities for infected and uninfected individuals in different landscapes vary (Jennelle *et al*. 2007).

Quantifying transmission rates of avian malaria, like many wildlife diseases, is difficult because, unlike for human diseases, individuals cannot all be counted or examined, and contact tracing (as is done for human diseases, such as SARS, see Lipsitch *et al*. 2003) is essentially impossible (McCallum, Barlow & Hone 2001; Caley & Hone 2004). Multistate mark–recapture models provide a framework for estimating epidemiologically relevant transmission parameters in natural populations, whilst explicitly accounting for variability in detection rates with infection status (Atkinson & Samuel 2010, Conn & Cooch 2009, Faustino *et al*., 2004, Schwarz, Schweigert & Arnason 1993). Transition rates obtained from multistate models are a compound measure of the probability of becoming infected and surviving to be captured and thus provide conservative estimates of infection rates, recovery rates and rates of change between different infections. In particular, transitions to infected states provide a conservative estimate of disease incidence rate (the discrete probability that susceptible individuals becoming infected during time i to $i + 1$, conditional on survival, Atkinson & Samuel 2010), which are related to the force of infection in the population (the rate at which susceptible hosts acquire infections, Heisey, Joly & Messier 2006; Ozgul *et al*. 2009). Although fast becoming an integral tool in wildlife disease ecology, such models have rarely been used to assess infection dynamics in avian malaria systems (Atkinson & Samuel 2010).

Another consideration in understanding malaria transmission in the wild is the variety of malaria species that may comprise infections in host populations (Bensch *et al*. 2004; Waldenström *et al*. 2004). Because both the prevalence and the distribution of different species may be governed by contrasting environmental conditions (Wood *et al*. 2007) and because different species might have quite different virulence in a given host (Lachish *et al*. 2011), species diversity can constitute a potentially important source of variation in risk of exposure and infection for hosts. To date, very few ecological studies of malaria have considered this diversity of malaria species with respect to infection dynamics.

In this study, we used multistate mark–recapture models to assess variation in infection dynamics of malaria in a long-term monitored population of blue tits (*Cyanistes caeruleus*) infected with two divergent *Plasmodium* species (*P. relictum* and *P. circumflexum*, Valkiūnas 2005). In a companion paper, we report the results of survival and recapture rate modelling from these multistate models, showing that these two malaria species impact on host fitness components in contrasting ways (Lachish *et al*. 2011). Here, we report the results of transition rate modelling using the same basic model structure to assess the patterns of infection and recovery rates within the population. Previous work has revealed marked variation in the spatial pattern of prevalence of these two malaria species within this population, in relation to key landscape (proximity to permanent water) and host characteristics (host age and sex, Wood *et al*. 2007). Hence, in this study, we aimed to (i) derive basic estimates of rates of infections and recovery; (ii) assess the role of these biological and environmental factors in forcing transmission processes within this population and (iii) determine whether the influence of biotic and abiotic factors on infection dynamics differs between malaria species.

Appendix 3

Materials and methods

Full details of the field and molecular diagnosis protocols used in this study are given in Lachish *et al.* (2011) along with fuller methods for the mark–recapture analyses. Here, we give brief details of these general methods and describe in full additional methods pertinent to the transition rate analyses presented in this paper.

STUDY SITE, HOST SPECIES AND AVIAN MALARIA DIAGNOSIS

From 2001 to 2009, blood samples were collected from individually marked blue tits (*Cyanistes caeruleus*) within Wytham Woods, near Oxford, UK (51°46′N, 1°20′W). Blood samples for diagnosis were collected annually between days 6 and 14 of the nestling phase, from parents feeding young in nest boxes. Blood samples were screened for infections of two *Plasmodium* morphospecies (based on cytochrome *b* sequences), *P. relictum* and *P. circumflexum* (Palinauskas *et al.* 2007), that comprise 98·2% of infections in the study population (Knowles *et al.* 2011). Infections were diagnosed either by nested polymerase chain reaction (PCR) assays from 2001 to 2004 (protocols described in Wood *et al.* 2007; Waldenström *et al.* 2004) or by quantitative (q)PCR assays from 2005 to 2009 (protocol details described in Knowles *et al.* 2011). For simplicity, in model notation, we refer to *P. relictum* as R-Clade and *P. circumflexum* as C-Clade (note that this is a change in terminology from previous work, e.g. Wood *et al.* 2007; Cosgrove *et al.* 2008; and reflects the identification of morphospecies that corresponded to distinct mtDNA lineages; Palinauskas *et al.* 2007).

MSMR MODELLING APPROACH

The mark–recapture data set consisted of yearly capture histories for all breeding birds captured within the study site each breeding season, grouped by sex and age and assigned to different disease states on the basis of their infection status at the time of capture. To incorporate individuals of unknown infection status [those for which blood samples were not obtained at capture, or for which analysis of blood samples was not carried out ($N = 1443$), for which species diagnosis was unresolved ($N = 46$), or for which diagnosis revealed mixed species infections ($N = 52$)] within our multistate mark–recapture framework, we employed newly developed 'multievent' models, an extension of multistate models (Pradel 2005; Conn & Cooch 2009). By explicitly accounting for unknown or partially observable states, by treating them as a hidden Markov process, multievent models allow for uncertainty in the detection of disease state, but not for error in the assignment of disease states. As discussed in Lachish *et al.* (2011), stringent laboratory procedures ensure that false-positive diagnoses will be rare in this study, although false-negative diagnoses may have occurred, as the majority of infections are chronic with low parasitaemia. However, analyses using occupancy modelling have shown the qPCR assay used in this study is highly sensitive, with a very low probability of false-negative diagnoses (S. Lachish, A. M. Gopalaswamy, S. C. L. Knowles & B. C. Sheldon, unpublished data). Moreover, when the probability of false positives is low and the true detection probability is at least 50%, then provided at least three samples are tested per unit (our qPCR diagnostic assays were run in triplicate) there will be very little bias in estimates (McClintock *et al.* 2010). The majority of diagnoses in this study were undertaken with qPCR in which samples were analysed in triplicate. Also, owing to the paucity of information on infection status in the early years of this study (only 58% of captured individuals were tested for malaria in 2001 and no individuals were tested in 2002) only two transition rate estimates were obtained in the years when nested PCR was used. Hence, we believe that the potential for biased estimates to produce spurious inferences in this study is minimal.

We conducted two multievent mark–recapture analyses to assess the infection dynamics of malaria in our study population. In the first analysis, we combined both *Plasmodium* species into a single infected state to assess the general patterns of infection and recovery within the population (see fig. 1a in Lachish *et al.* 2011). Capture histories for this analysis were assigned to one of three events (captured and infected, captured and uninfected, captured but infection status unknown) corresponding to two disease states (infected and uninfected). All state transitions were possible, as infected individuals can recover, and recovered individuals may become re-infected, with yearly infection and recovery rates given by the transitions from uninfected to infected states and from infected to uninfected states, respectively. In the second analysis, we kept both *Plasmodium* species separate to assess species-specific infection dynamics and assigned capture histories to one of four events (captured with R-Clade infection, captured with C-Clade infection, captured and uninfected, captured but infection status unknown), corresponding to three disease states (uninfected, infected with R-Clade, infected with C-Clade; see fig. 1b in Lachish *et al.* 2011). Again all transitions between states were possible, with transition rates now representing species-specific infection and recovery rates and rates of switching between infection types. Because acute malaria infections appear to entail substantial mortality costs for hosts in this population (Lachish *et al.* 2011), transition rates estimated in this study will largely reflect infection dynamics amongst uninfected and chronically infected individuals. In addition, as a consequence of *Plasmodium* epidemiology (Valkiūnas 2005), annual estimates of infection rates will capture both new infections and relapses of previous infections, whilst annual estimates of recovery will comprise both true recovery (sterilizing immunity) and apparent recovery (involving the disappearance of active blood-stage infections when infections remain latent in tissues or are suppressed below the diagnostic detection limit; see also Discussion).

For each analysis, we employed a three-stage model ranking process. We first modelled recapture rates with survival and transition rates fully parameterized (see Lachish *et al.* 2011 for details of the global model). Survival rates were then modelled using the most parsimonious recapture rate model identified in step one. The results of these recapture and survival rate models are reported elsewhere (Lachish *et al.* 2011). Here, we report the final stage in the MSMR model process: modelling variation in transition rates, using the most parsimonious recapture and survival models identified in the first two stages as the base models for this final stage. For the combined *Plasmodium* analysis, the base model for recapture rate was state and sex dependent (St + Sx), for survival rate was age (first year birds vs. adults of 2+ years of age) and sex dependent (a2 + Sx) and allowed for temporal variation in all transition rates as well as age and sex effects [(St*t + a2 + Sx); QAIC = 7525·71; see Table 1 for notation and table 3 in Lachish *et al.* in press for model selection results]. For the separate-species analysis, the base model for recapture rate was state and nest box density-dependent (St + boxD), with survival modelled as state, time and age dependent (St + t + a2) and allowed for temporal variation in all transition rates (except those occurring between R-Clade and C-Clade as these transitions were infrequent in our data set) as well as age and sex effects [(St*t + a2 + Sx); QAIC = 7141·21 see table 4 in Lachish *et al.* 2011]. Whilst the modelling process for this paper is an extension of

Table 1. Notation used to denote the main effects and model structure used in modelling transition rates (Ψ) of blue tits infected with *Plasmodium* species (*P. relictum* = R-Clade; *P. circumflexum* = C-Clade)

Model notation	Description
St	State-dependent transition rates (indicates that all transition rates differ and covariate effects apply to all transition rates)
Inf	Infection rates (indicates that covariate effects are limited to transitions from uninfected to infected states: Ψ_{UI} for the clades combined analysis, or Ψ_{UR} and Ψ_{UC} for the separate clades analysis)
- (RInf; CInf)	Clade-specific infection rates (covariate effects limited to Ψ_{UR} or Ψ_{UC}, respectively)
Rec	Recovery rates (indicates that covariate effects are limited to transitions from infected to uninfected states: Ψ_{IU} for the clades combined analysis, or Ψ_{UR} and Ψ_{UC} for the separate clades analysis)
- (RRec; CRec)	Clade-specific recovery rates (covariate effects limited to Ψ_{RU} or Ψ_{CU}, respectively)
RC	Transition from R-clade infection to C-clade infection
CR	Transition from C-clade infection to R-clade infection
Covariate effects	
Sx	Sex effect
a2	Age effect (yearlings vs. adults)
river	Distance from the River Thames (dichotomous near/far covariate)
d2river	Distance from the River Thames (continuous individual covariate)
t	Time dependence (yearly variation)
c	Constant rate (no covariates)

the previous work, the question (dynamics of infection vs. effects on hosts) is different, and separation of the two allows more detailed dissection of the effects.

To assess the relative influence of environmental and host factors in mediating transmission dynamics and infection risk within the host population, we modelled variation in transition rates in two steps. Based on *a priori* knowledge, we first assessed the importance of temporal and environmental variation relative to constant transition rates by modelling transition rates in relation to time (year) and proximity to a major water body, the River Thames (which are known to be correlated with *Plasmodium* prevalence in this population, Wood *et al.* 2007). Proximity to the river was included in models either as: (i) a dichotomous covariate, with individuals classified as being either near the river (captured in nest boxes that were ≤500 m from the river) or far from the river (captured > 500 m from the river) based on previous work showing higher *Plasmodium* prevalence within this distance of the River Thames (Wood *et al.* 2007); or as (ii) a continuous individual-specific covariate, with the (standardized) distance to the river determined by GIS from the nest box in which individuals were first captured breeding. We investigated the additive and two-way interactive effects of time and river on state transitions (including state*time and state*river interactions with transition rates only). However, as mentioned earlier, in the species-specific analysis, changes in infection status from R-Clade to C-Clade infection or vice versa were constrained to be time-invariant, as these transitions were infrequent in our data set. Also, to limit the number of candidate models in the species-specific analysis, the 'continuous' river covariate was only modelled as an alternative for the 'dichotomous' river effect in the best identified model (and was not included in further models, as it did not improve the fit of this model, see Results). In the second step, we assessed variation in transition rates in relation to host sex and age, whilst retaining the temporal and river effects of the best model in the previous step. We investigated the additive and two-way interactive effects of age and sex on state transitions (including state*age and state*sex interactions only). Note that in the species-specific analysis, the effects of host age and sex were not modelled for transitions that occurred between R-Clade and C-Clade infections.

All models were fitted to the data using program E-SURGE (Choquet 2009) using the general model structure described in Conn & Cooch (2009; specific details given in Lachish *et al.* 2011). Model selection was based on small sample size corrected Akaike Information Criteria adjusted for overdispersion (QAICc with $\hat{c} = 1{\cdot}5$; see Lachish *et al.*, 2011, for details of goodness-of-fit procedures for global models). ESURGE automatically adjusts AIC values and parameter variances to account for this variance inflation factor. The relative likelihood of each model in the candidate set was estimated with normalized QAICc *w*eights (w_i, or the index of relative plausibility). Model notation is explained in Table 1.

Results

A total of 3424 birds were captured an average of 1·4 times for a total of 4843 captures over the 9 years of the study (complete summaries of mark–recapture data provided in Appendix S1). As no information on infection status was available for individuals captured in 2002, transition rates between all states in both analyses were inestimable in the early years of the study [from 2001 to 2003; confidence intervals for these estimates were either abnormally large (from 0 to 1) or small (equal to zero)]. As these early years contributed to robust inferences of survival and recapture rates in this population (see Lachish *et al.* 2011), they were retained in the model structure, and, however, their exclusion did not qualitatively change results. From 2004 to 2009, we observed 175 transitions between known disease states in the combined-species analysis and 167 transitions amongst known disease states (the uninfected and the two infected states) in the species-specific analysis (all transitions including those involving unknown disease states were more numerous: 381 for the combined analysis and 354 for the clade-specific analysis).

The combined-species analysis revealed very strong support for models in which transition rates varied between years and in relation to whether individuals were near or far from the river (i.e. treated as a dichotomous variable), indicating that both the disease incidence rate and the likelihood of recovery vary temporally within the population, and

Table 2. Summary results of the multievent mark–recapture analysis modelling transition rates of combined *Plasmodium* infections in blue tits. The top 10 models in each modelling step are shown (as well the model with constant transition rates). Recapture rate was modelled as state and sex dependent (St + Sx), whilst survival was modelled as age and sex dependent (a2 + Sx) based on results from initial models of survival and recapture effects (with the base model shown in bold; see Lachish *et al.* 2011 for results of recapture and survival rate modelling)

Modelling step	Model[a]	K[b]	Deviance	QAICc	ΔQAICc	w_i[c]
(a) Temporal and environment effects	St*t + St*river[d]	32	11 183·38	7520·05	0	0·793
	St*t + Inf_{river}	31	11 190·94	7523·07	3·019	0·176
	St*t + St*d2river	32	11 200·79	7526·548	6·489	0·031
	Inf_t + Rec_c	23	11 232·89	7534·84	14·781	0·000
	St*t	30	11 212·32	7535·29	15·236	0·000
	St*t + river	31	11 210·65	7536·21	16·151	0·000
	St*river + t	25	11 212·80	7539·67	19·614	0·000
	St*t + d2river	31	11 213·67	7547·90	27·841	0·000
	St*d2river + t	25	11 231·54	7547·92	27·870	0·000
	Inf_c + Rec_t	23	11 231·31	7547·95	27·896	0·000
	Inf_c + Rec_c	16	11 273·67	7547·96	27·900	0·000
(b) Age and sex effects	St*t + St*river	32	11 183·38	7520·05	0	0·190
	St*t + St*river + Inf_{a2}	33	11 180·64	7520·26	0·200	0·171
	St*t + St*river + Rec_{a2}	33	11 181·32	7520·71	0·656	0·136
	St*t + St*river + Inf_{Sx}	33	11 181·92	7521·12	1·058	0·112
	St*t + St*river + Inf_{a2} + Rec_{a2}	34	11 178·94	7521·16	1·102	0·110
	St*t + St*river + $Inf_{a2 + Sx}$	34	11 179·22	7521·35	1·287	0·100
	St*t + St*river + Inf_{a2} + Rec_{Sx}	34	11 180·50	7522·20	2·142	0·065
	St*t + St*river + Inf_{Sx} + Rec_{Sx}	34	11 190·94	7523·07	3·012	0·042
	St*t + St*river + $Inf_{a2 + Sx}$ + $Rec_{a2 + Sx}$	36	11 181·87	7523·11	3·055	0·041
	St*t + a2 + Sx	**32**	**11 204·02**	**7525·71**	**5·652**	**0·011**

[a]Model notation described in Table 1. * indicates interaction between variables; + indicates additive effects; covariates in subscripts pertain only to the transition denoted; other covariates pertain to all transitions.
[b]Number of parameters.
[c]Model weight.
[d]Most parsimonious model from step 1 retained for modelling age and sex effects in step 2.

spatially within the study site (Table 2a). Models in which distance to the river was included as an individual covariate were not well supported by the data (ΔQAICc = 6·49 between the best-supported model and the highest ranked model with river as an individual covariate). Estimates from the best-supported model in this stage of the analysis (with 79% of the weight in the candidate set) show that infection rates were on average 55% higher and recovery rates 57% lower in areas located near the River Thames (Fig. 1). This figure also demonstrates the temporal variability in overall infection and recovery rates within the population, with infection and recovery negatively correlated and alternating between higher and lower rates in consecutive years (Fig. 1). The results of model selection examining the effects of host age and sex on infection dynamics revealed that models in which infection and recovery rates varied with host age or sex were amongst the top models but were not better supported than the model with only time dependence and variation in relation to distance to the river (Table 2b). As there was very little difference in the degree of support for the top models in this candidate set, the relative influence of these biotic and abiotic factors on either infection or recovery rates could not be determined from this analysis.

In the species-specific analysis, model selection again revealed very strong support for models in which transition rates varied in relation to the distance from the river (Table 3a), highlighting the importance of this landscape feature for driving variation in the disease incidence rate in the population and influencing the risk of infection for individuals. However, in this analysis, proximity to the river was found to influence only those transition rates which involved C-Clade (*P. circumflexum*) infections: C-Clade infection rate,

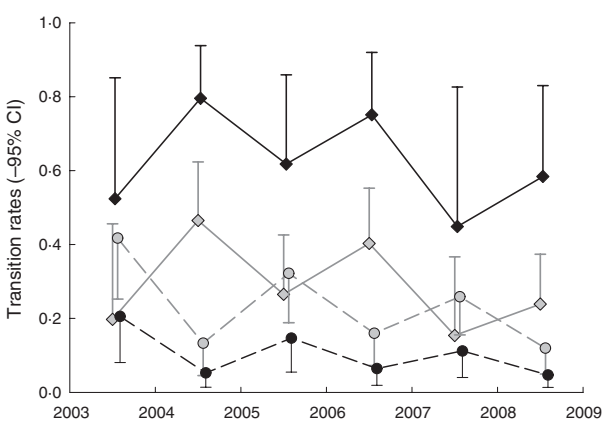

Fig. 1. Estimates (±95% CI) from the best-supported model in step 1 of the combined *Plasmodium* multievent mark–recapture analysis showing transition rates of blue tits over the duration of the study in relation to proximity to the River Thames. Infection rates are shown in solid lines; recovery rates are shown in dashed lines; transition rates close to the river shown in black; transition rates far from the river shown in grey. To clarify patterns, not all error bars are shown.

Table 3. Summary results of multievent mark–recapture analysis modelling transition rates of *Plasmodium relictum* (R-Clade) and *Plasmodium circumflexum* (C-Clade) infections in blue tits. The top 10 models in each modelling step are shown (as well the model with constant transition rates). Recapture rate was modelled as nest box density- and state dependent (St + boxD), whilst survival was modelled as state, time and age dependent (St + t + a2; with the base model shown in bold; see Lachish *et al.* 2011)

Modelling step	Model[a]	K[b]	Deviance	QAICc	ΔQAICc	w_i[c]
(a) Temporal and environmental effects	$[RC + CR + CInf + CRec]_{river} + RInf_t + RRec_c$[d]	40	10 562·49	7122·64	0	0·972
	$[RC + CR + CInf + CRec]_{river} + Inf_t + RRec_c$	47	10 551·98	7130·01	7·3619	0·024
	$[RC + CR + CInf + CRec]_{river} + Inf_t + Rec_t$	61	10 514·78	7134·13	11·491	0·003
	$[RC + CR + Inf + Rec]_{river} + Inf_t + Rec_t$	63	10 513·72	7137·58	14·937	0·001
	$[RC + CR + RInf + RRec]_{river} + Inf_t + Rec_t$	61	10 524·43	7140·56	17·920	0·000
	$[RC + CR + CInf + CRec]_{d2river} + RInf_t + RRec_c$	36	10 598·24	7143·28	20·640	0·000
	$[RC + CR + CInf + CRec]_{river} + RInf_c + Rec_t$	47	10 573·86	7144·59	21·950	0·000
	$RC_c + CR_c + Inf_c + Rec_t$	43	10 587·38	7147·32	24·681	0·000
	$RC_c + CR_c + CInf_c + RInf_t + Rec_c$	36	10 613·70	7148·59	25·952	0·000
	$RC_c + CR_c + Inf_t + Rec_c$	43	10 596·69	7151·59	28·952	0·000
	$RC_c + CR_c + Inf_c + Rec_c$	29	10 655·82	7161·88	39·240	0·000
(b) Age and sex effects	$[RC + CR + CInf + CRec]_{river} + RInf_{t+a2} + CInf_{Sx} + RRec_c$	42	10 548·45	7117·38	0	0·465
	$[RC + CR + CInf + CRec]_{river} + RInf_{t+a2} + RRec_c$	41	10 555·40	7119·96	2·580	0·128
	$[RC + CR + CInf + CRec]_{river} + RInf_{t\ +\ Sx} + CInf_{Sx} + RRec_c$	42	10 553·26	7120·58	3·208	0·094
	$[RC + CR + CInf + CRec]_{river} + RInf_{t+a2} + Rec_{Sx}$	42	10 555·10	7121·81	4·435	0·051
	$[RC + CR + CInf + CRec]_{river} + RInf_{t+a2+Sx} + RRec_c$	42	10 555·20	7121·88	4·503	0·049
	$[RC + CR + CInf + CRec]_{river} + RInf_t + Inf_{a2} + RRec_c$	41	10 558·35	7121·93	4·549	0·048
	$[RC + CR + CInf + CRec]_{river} + RInf_{t+} + Inf_{a2} + Rec_{Sx}$	42	10 555·86	7122·32	4·942	0·039
	$[RC + CR + CInf + CRec]_{river} + RInf_t + Inf_{a2+Sx} + RRec_c$	42	10 556·24	7122·57	5·190	0·035
	$[RC + CR + CInf + CRec]_{river} + RInf_t + Inf_{a2} + Rec_{a2}$	42	10 556·36	7122·66	5·274	0·033
	$[RC + CR + CInf + CRec]_{river} + RInf_t + Rec_{a2}$	41	10 560·04	7123·06	5·674	0·027
	$\mathbf{RC_c + CR_c + Inf_t + Rec_t + Inf_{a2+Sx} + Rec_{a2\ +\ Sx}}$	**57**	**10 562·61**	**7141·21**	**4·108**	**0·000**

[a]Model notation described in Table 1.* indicates interaction between variables; + indicates additive effects; covariates in subscripts pertain only to the transitions denoted (and to all transition rates listed within square brackets).
[b]Number of parameters.
[c]Model weight.
[d]Most parsimonious model from step 1 retained for modelling age and sex effects in step 2.

C-Clade recovery rate and transitions between C-Clade and R-Clade (Table 3a). Models in which R-Clade (*P. relictum*) infection or recovery rates varied with proximity to the river received little support by the data. Again in this analysis, models with distance to the river included as an individual covariate were not well supported by the data (Table 3a). Estimates from the best-supported model at this stage of the analysis (with 97% of the weight in the candidate set) show that C-Clade infection rates were on average 75% greater near the river than further away, whilst recovery rates from C-Clade infections were on average 55% lower near the river than further away (Fig. 2). This model also revealed that transitions from R-Clade to C-Clade infections occurred almost exclusively near the river, whereas transitions from C-Clade to R-Clade infections occurred more often amongst individuals located far from the river (Fig. 2).

Examining infection dynamics separately for the two malaria species also proved valuable for elucidating patterns of temporal variability in transition rates. In the separate-species analysis, the best-supported model in the first stage of analysis included temporal variation only for R-Clade infection rates (Table 3a). R-Clade infection rates alternated between higher and lower values in consecutive years (Fig. 3a), suggesting that the temporal variation observed in the overall disease incidence rates in the population is largely driven by temporal variation in R-Clade infection rates.

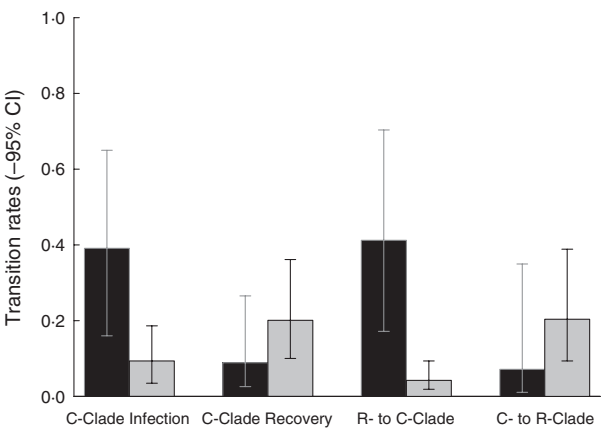

Fig. 2. Estimates (±95% CI) from the best-supported model in step 1 of the species-specific multievent mark–recapture analysis showing transition rates of blue tits infected with *Plasmodium circumflexum* (C-Clade) as well as transition rates between *Plasmodium relictum* (R-Clade) and *P. circumflexum* (C-Clade) infections in relation to proximity to the River Thames (transition rates close to the river shown in black; transition rates far from the river shown in grey).

Incorporating species information on infection status likewise afforded us a clearer understanding of the effects of host age and sex on infection dynamics. In this analysis, model selection revealed substantial support for models in which infection rates, but not recovery rates, varied with host age or

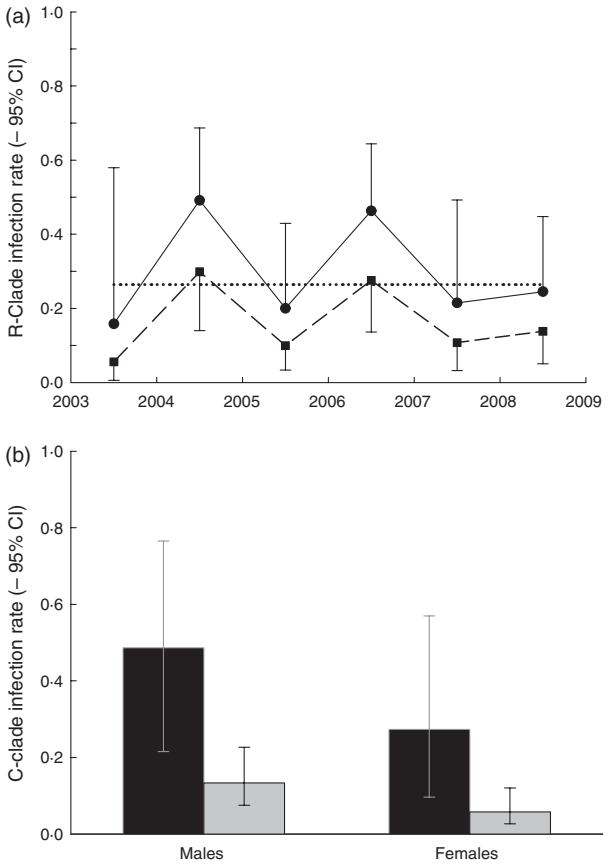

Fig. 3. Results of species-specific mark–recapture analysis showing (a) the effect of age (circles and solid lines for yearlings; squares and dashed lines for adults) on the infection rates of blue tits with *Plasmodium relictum* (R-Clade), as well as the recovery rate from R-Clade infections for blue tits (shown as dotted line); and (b) the effect of sex on infection rates of blue tits with *Plasmodium circumflexum* (C-Clade) as a function of proximity to the river (close to the river shown in black; far from the river shown in grey). Estimates are model-averaged means (±95% CI). To clarify patterns, not all error bars are shown.

sex (Table 3b), suggesting that these biotic traits influence the rate at which individuals become infected, but not the rate at which they clear infections. Furthermore, the model selection process revealed that the effects of these biological traits on infection rates differed between the two malaria species. The best-supported model in the candidate set showed that C-Clade infection rates were influenced by host sex alone, whilst R-Clade infection rates were influenced only by host age (Table 3b). Model-averaged estimates show that R-Clade infection rates were higher for adults than for yearlings (Fig. 3a), whilst C-Clade infection rates were higher for males than for females, regardless of proximity to the river (Fig. 3b).

Discussion

Using a novel multievent mark–recapture framework (Choquet 2009; Conn & Cooch 2009) to account for infection state–dependent differences in host detectability, we found evidence of striking spatial heterogeneity in malaria transmission rates within a single population of blue tits over a small spatial scale. Moreover, we found marked differences in the role of environmental and host factors in forcing infection dynamics of the different malaria species within this population and also documented distinct patterns of temporal variation in infection dynamics of these two malaria species. Thus, this study documents that the processes causing infection by pathogens in natural populations are far from homogenous in space or time and also demonstrates that knowledge of the drivers of spatial and temporal heterogeneity in disease transmission will be crucial for developing accurate epidemiological models and a thorough understanding of the evolutionary implications of pathogens. The scale over which these variable effects were demonstrated is rather small; individuals living a few hundred metres apart (which is considerably less than the median natal dispersal distance in this population; S. C. L. Knowles & B. C. Sheldon, unpublished) were subject to quite different forces of infection as well exposure to potentially different pathogens.

Abiotic factors have been shown to play a particularly prominent role in driving the transmission dynamics of vector-borne pathogens (Randolph 2001; Byers *et al*. 2008; Borer *et al*. 2010), including malaria (Foley *et al*. 2003; Balls *et al*. 2004). Environmental drivers of malaria transmission include altitude, climatic conditions and proximity to permanent water, factors that can either limit the abundance and distribution of vectors or impinge on parasite vigour (Foley *et al*. 2003; Balls *et al*. 2004; Freed *et al*. 2005; Pope *et al*. 2005; Atkinson & LaPointe 2009). In this study, we found that proximity to the River Thames, the only large permanent water source in or near to our study site, influenced the transmission rates of *P. circumflexum*, but not of *P. relictum*. Our results revealed that this was not a monotonic linear effect with increasing distance from the river but better described as a dichotomous effect of proximity to permanent water *per se*. Indeed, the disease incidence rate of *P. circumflexum* was nearly four times greater and consequently, the likelihood of recovery substantially lower in areas within 500 m of the river than in areas further away (explaining the similar pattern observed for combined *Plasmodium* transmission rates). This result clearly indicates that proximity to the river is a key determinant of host infection risk for *P. circumflexum* in this population. This result is consistent with the expectation that *P. circumflexum* and *P. relictum* are transmitted by different vectors, with the mosquito species responsible for transmitting *P. circumflexum* being restricted within the study site by the availability of wet larval habitat for breeding. Preliminary investigations into mosquito ecology at our study site have found a total of 14 mosquito species, or species complex, with overall mosquito abundance varying spatially through the study site (R. Alves, M. J. Wood, C. Cowell, B. C. Sheldon, unpublished data). The most abundant species in the area near the River Thames, *Ochlerotatus annulipes* (R. Alves, unpublished data), lays its eggs exclusively on damp soil or leaf litter (Cranston *et al*. 1987) and is a potential candidate vector for *P. circumflexum*

transmission. Clearly, further investigation into the biology and ecology of vectors within our study site, and particularly their vector competency with respect to the different avian malaria species, is needed to be able to verify this suggestion.

Infection rates of *P. relictum* amongst birds, although not influenced by proximity to the River Thames, did display marked variation between years in this study and were the primary driver of similar temporal variation observed in overall disease incidence rates of *Plasmodium* in the population. Although these two processes need not be correlated, the observed pattern of variation in infection and recovery rates between years may be explained solely by changes in the underlying force of infection in the population. A high force of infection will result in many new infections in hosts, but few observed recoveries, whilst more recoveries and fewer new infections will be observed when the force of infection is low. One potential explanation for this marked annual variation in the underlying force of infection in the population is that the vector or vectors responsible for *P. relictum* transmission fluctuate in abundance according to annual climatic variation (e.g. temperature and rainfall), which alter the microhabitat or microclimates they require for breeding. Greater transmission rates may thus occur in years when conditions are more favourable for vectors. Alternatively, annual variation in host demography and population dynamics could also play a role in driving this temporal variability, via periods of greater or lower immigration or recruitment of immunologically naive individuals (Anderson & May 1986; Atkinson & Samuel 2010). However, the effects of annual variation in host demography and population dynamics would presumably also be expected to affect infection dynamics of other *Plasmodium* species, and very little temporal variation was observed for *P. circumflexum* transmission. Again, an understanding of the life cycles and behaviour of the mosquito species in our study system and their vector competency would assist in explaining the observed pattern of temporal variation in disease incidence rates and of *P. relictum*.

Although host factors such as age and sex are known to predict the prevalence of malaria infection in a variety of avian hosts, although not always in a consistent manner (Korpimäki, Hakkarainen & Bennett 1993; Marzal *et al*. 2008; van Oers *et al*. 2010), few ecological studies have yet explored their influence on transmission in the wild (Atkinson & Samuel 2010). In this study, we found that malaria infection rates, but not recovery rates, were influenced by host age and sex, but that the manner in which these biotic factors influence transmission differed between the two *Plasmodium* species. Infection rates for *P. circumflexum* were greater for males than for females. This pattern might result from differences in life history (e.g. if males settle earlier on breeding territories and are thus exposed for longer) or reproduction or foraging behaviours (e.g. males do not incubate or may spend more time foraging) causing male blue tits to experience greater exposure to vectors of this parasite species. Such differences in life history may not have resulted in detectable differences in *P. relictum* infection rates for males and females, because *P. relictum* is more patchily distributed within the study site than *P. circumflexum* (Wood *et al*. 2007). More detailed analysis of individual variation in settlement time or foraging behaviour might shed light on these hypotheses. Infection rates for *P. relictum* did vary between yearlings and adults, however, with adults being more likely to acquire infection over a yearly interval. Whilst it is possible that a difference in the length of exposure for adults and yearlings could affect the rate at which they acquire infections, it is difficult to understand why this would only manifest in an effect for *P. relictum* and not *P. circumflexum*. In addition, this pattern cannot be explained by greater survival prospects for infected adults, as the previous work has shown that infected adults have lower survival rates than infected first years (Lachish *et al*. 2011). Although we can only speculate at present, age-specific differences in immune function (as it relates to the likelihood of infection relapse) could play a role in driving these differences in transmission rates (Wood *et al*. 2007; van Oers *et al*. 2010). Clearly, further work is needed to clarify the physiological and ecological mechanisms by which age and sex influence transmission rates of different *Plasmodium* species.

One limitation of the assessment of the role of biotic and abiotic factors in driving malaria infection dynamics in this study is that infection status was only tested annually during the host's breeding seasons. The timing of malaria transmission in this population is not precisely known, but preliminary data suggest that the majority of infections may be acquired after the breeding season in this population, in mid to late summer (when vectors are expected to be most abundant and also when immunologically naive juvenile enter the host population), with negligible transmission assumed in winter (as vector activity wanes and parasites disappear from the blood; Cosgrove *et al*. 2008). Thus, not only are we likely to have missed many transitions amongst infection states, but our transition rate estimates clearly reflect the combined effect of these varied seasonal dynamics on infection and recovery processes. Further investigation into the drivers of transmission dynamics would help to clarify to what extent infections represent new infections or relapses of previous infections, and to what extent host vs. environmental factors drive recovery (infection loss) rates outside of the winter period. However, as the seasonal pattern of malaria transmission still needs to be characterized, there remain significant gaps in our understanding of the infection dynamics of avian malaria in natural populations.

Another challenge in relating estimates of transition rates obtained in this analysis to true infection rates in the population is that our previous work has shown that disease-induced mortality differs between the two parasite species (Lachish *et al*. 2011). Differences in disease-induced mortality of hosts will cause transition rate estimates to be biased to differing degrees for each of the *Plasmodium* species. Infection rates for *P. circumflexum* (which substantially reduced host survival relative to *P. relictum*) will have been underestimated in this study, whereas infection rates for *P. relictum* will have been estimated with little bias, as the negative

impacts of *P. relictum* infection on host reproductive output seemed, if anything, to benefit (improve) host survival prospects (Lachish *et al*. 2011). Nevertheless, as even biased transition rates still provide a conservative (under) estimate of underlying variation in disease transmission, they can still contribute to inference on infection dynamics in this system, though care is needed in their interpretation, as the magnitude of the relative rates may not reflect reality.

One further caveat to the inferences drawn in this study is that the multievent mark–recapture model, like most standard multistate analyses, assumes that state transitions are first-order Markovian, such that the probability of an individual making a transition between time i and $i + 1$ depends only on its state at time i (Williams, Conroy & Nichols 2001; Pradel 2005). The possibility of relapses of prior infections, as well as differences in host-acquired immunity to infection, implies that this may be a naive assumption for this disease system. Extensions of multievent models allow state transitions to be modelled as higher-order Markovian process (so-called memory models, as per Hestbeck, Nichols & Malecki 1991). Although these memory models will be critical for elucidating the role of host immune response and host genetic factors in the transmission dynamics of diseases like avian malaria, they demand vast amounts of data and are thus difficult to implement in reality. Certainly, the sparseness of the relevant aspects of our data (over 9 years only 175 transitions between known disease states in 4843 capture of 3424 individuals) prevented us from modelling state transitions as a higher-order Markov process.

Investigating temporal and spatial patterns of infection dynamics in wild populations can inform about the role of environmental factors in mediating host–pathogen interactions. In this study, we documented striking spatial variation in the transmission dynamics of two avian malaria species within a single population of blue tits. This indicates that landscape variables are capable of driving avian malaria transmission at much smaller spatial scales than previously recognized (Perez-Tris & Bensch 2005; Atkinson & Samuel 2010; Loiseau *et al*. 2010). The fact that these differences in transmission intensity occur at a local scale (over a few hundred metres) also suggests that the selective effects of malaria infection on avian hosts can be very different over very small spatial scales. Consistent differential selection pressures for different genetic aspects of host resistance in spatially segregated areas can lead to local adaptation of hosts and parasites (Dybdahl & Storfer 2003). Although spatial variation in the disease incidence rate of *P. circumflexum* in this population was shown to be stable over the study, dispersal and immigration rates are high in the population, diminishing the potential for host–parasite co-evolution. Nonetheless, if dispersal is non-random with respect to resistance phenotype, then local adaptation might occur even in the face of marked dispersal (see Garant *et al*. 2005 for an example of this process in a different context). On the other hand, because proximity to the River Thames was influential for determining overall risk of malaria infection in this population, and infection carries substantial fitness costs for hosts (Lachish *et al*. 2011), the potential for host dispersal to mediate the impacts of infection for hosts is evident. Further investigation as to whether differential dispersal in juveniles and adults can reduce subsequent parasitism rates and lead to optimal dispersal strategies for disease avoidance in this system is clearly warranted. Overall our findings demonstrating that the role of environmental and host factors in driving patterns of avian malaria transmission can vary dramatically between different *Plasmodium* species suggest that future attempts to understand the epidemiology, ecology and evolutionary implications of avian malaria parasites in wild populations should account not only for temporal and spatial variation in factors affecting vector distribution and abundance and host demography, but also the diversity of haematozoan species present within the host population.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Appendix S1. M-arrays providing a concise summary of the mark–recapture datasets used in multi-event modelling of the infection dynamics of (a) combined *Plasmodium* infection in wild blue tits; and (b) species-specific *Plasmodium* infection dynamics.

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Spatial determinants of infection risk in a multi-species avian malaria system.

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ABSTRACT

Spatially-variable processes can be an important element of host-parasite interactions, but their longer term demographic and evolutionary effects depend on the magnitude of variation in space, the scale at which variation occurs and the degree to which such processes are temporally stable. Here, we use multiple years of data from a study of two closely related tit species (*Paridae*), infected with two congeneric species of avian malaria (*Plasmodium*), to evaluate the roles of extrinsic and intrinsic factors in driving spatial heterogeneity in infection risk, and to address questions of scale and temporal stability in these vector-driven host-parasite interactions. We show that the two malaria parasite species exhibit markedly different spatial epidemiology: *P. relictum* infections are effectively randomly distributed in space, with no temporal consistency, whereas *P. circumflexum* infections exhibit pronounced spatial structuring that is stable over the six years of this study and similar in both host species. We show that both conspecific and heterospecific host density contribute to elevated infection risk, but that the main determinants of elevated risk of *P. circumflexum* infection risk are habitat features probably associated with vector distribution and abundance. We discuss the implications of these findings, both for our understanding of the epidemiology of malaria in the wild, but also in terms of the longer-term evolutionary and demographic consequences that spatially variable parasite-mediated selection may have on host populations.

INTRODUCTION

Spatial heterogeneity shapes many ecological processes, including host–parasite interactions (Ostfeld et al. 2005). As parasitism represents a major selective force for wild animal populations, spatial heterogeneity in host-parasite interactions will affect not only epidemiology, but also the strength and specificity of selection pressures on hosts, as well as the evolutionary effects of selection (Poulin 2007, Foster et al. 2007, Wolinska and King 2009). Hence, to thoroughly understand both disease ecology and the evolutionary implications of pathogens for hosts it is necessary to characterise spatial variation in infection risk and identify the factors that determine the distribution of parasite infections in wild populations.

Several processes can generate spatial heterogeneity in infection risk for wild populations. When parasite dispersal is localised, or transmission varies as a function of host population density, then aggregations in the distribution of hosts can lead to strong spatial variation in disease risk (Cross et al. 2008). Because many pathogens can infect multiple host species, each of which may vary in its transmission competency, spatial variation in the relative abundance of different hosts is also likely to be important in generating spatial variation in infection risk (States et al. 2009). Additionally, aggregations of hosts with differing degrees of susceptibility to infection can also result in spatial variation in infection risk (Real and Biek 2007). Similarly, factors such as body condition, or stress, that influence the risk of parasite infection for hosts, can also vary spatially as a result of differences in habitat quality (Beldomenico et al. 2009) or as a function of conspecific or heterospecific density. In addition to these intrinsic host factors, extrinsic abiotic factors such as habitat characteristics and climatic conditions may constrain the distribution of pathogens, hosts, reservoirs, or

vectors, thus placing spatial constraints on the geographic extent of host-pathogen interactions (Loiseau et al. 2010, Sehgal et al. 2011).

Avian malaria parasites (*Plasmodium spp*) (Valkiūnas 2005) are globally distributed vector-borne parasites. The spatial dependence of malaria transmission is well recognised at scales relating to geographically separate host populations and across biogeographical regions (Bensch et al. 2007, Loiseau et al. 2010, Sehgal et al. 2011). Heterogeneity in habitat and landscape characteristics on a local scale can strongly influence the spatial and temporal distribution of favourable conditions for vector proliferation (Wood et al. 2007), and hence might cause small-scale variation in malaria infection risk. However, the majority of studies investigating the spatial dynamics of malaria in avian hosts to date have been conducted at large spatial scales (Scheuerlein and Ricklefs 2004). Moreover, the role of intrinsic versus extrinsic drivers of spatial variation in malaria risk can differ at different scales (Ostfeld et al. 2005). For example, studies in human malaria systems (*P.falciparum*) have shown that while environmental conditions generate spatial patterns of infection at large spatial scales (>100km), variation in infection risk at a local scale (<5km) is often strongly driven by intrinsic population-related processes (Grillet et al. 2010, Silué et al. 2008). For these reasons, the extent and causes of spatial variation in malaria infection risk at a local scale are still poorly understood for wild populations.

Our understanding of spatial variation in infection risk in avian malaria systems is also limited by the paucity of longitudinal data on infection in natural populations. Wild birds are rarely re-sampled, yet only long-term individual-level data can reveal how stable spatial variability in infection risk is over time (Bensch et al. 2007). Such information is important for understanding disease dynamics and assessing the potential for local adaptation and host-

parasite co-evolution. Further complexity is introduced by the fact that infections in host populations may comprise several malaria parasite species, each infecting multiple hosts (Bensch et al. 2004); few studies of avian malaria have compared spatial patterns of infection risk for sympatric host species infected by multiple parasite lineages.

Long-term geo-referenced datasets on avian malaria in wild populations that incorporate ecologically relevant multi-host-pathogen complexities offer excellent opportunities to investigate the factors that shape spatial variation in infection risk on a local scale, and host-parasite co-evolutionary dynamics in natural populations. Two recent studies (Knowles et al. 2011, Wood et al. 2007) used a geo-referenced dataset to investigate variation in malaria infection within a long-term monitored population of blue tits, *Cyanistes caeruleus*, infected with two divergent *Plasmodium* species (*P. relictum* and *P. circumflexum*, Valkiunas 2005). These studies reported spatial differences in the distribution of the two malaria parasite species, attributed to interactions between local landscape features, host factors and parasite species (Knowles et al. 2011, Wood et al. 2007). Here we expand on this dataset to evaluate the relative roles of extrinsic and intrinsic factors as drivers of the long-term spatial dynamics of *Plasmodium* infections. We significantly broaden the scope of previous studies by: (i) examining the temporal consistency of spatial variation in *Plasmodium* infection risk within this single woodland site over six years; (ii) by comparing spatial patterns of infection risk with the two *Plasmodium* species between blue tits and a second sympatric host species, great tits (*Parus major*), and by (iii) employing spatially-explicit statistical methods to characterise spatial heterogeneity in infection risk, identify local clusters of infection, assess the spatial scale of disease risk, and evaluate the role of environmental and host factors in driving spatial variation in disease risk.

METHODS

Study site, host species and avian malaria diagnosis

Approximately 1160 nestboxes are monitored in Wytham Woods, a 385-ha woodland near Oxford, UK (51°46′N, 1°20′W), where between 250 and 450 pairs of blue tits (*Cyanistes caeruleus*) and great tits (*Parus major*) breed annually. From 2005 to 2010 blood samples for infection diagnosis were collected from individually marked blue tits between day 6 and 14 of the nestling phase. In 2008 and 2009 blood samples were also obtained from individually marked great tit parents at the same standardised point in their breeding cycle. As these populations are single brooded and breeding is synchronous, there is relatively little variation in the calendar date among samples within each year (average range ± SE = 42.42 ± 6.78 days). Blood samples were screened via quantitative (q)PCR for infection by two *Plasmodium* species, *P. relictum* and *P. circumflexum*, (Palinauskas et al. 2007), that comprise 98.2% of infections in the study population (Knowles et al. 2011, Lachish et al. 2011; mixed species infections were rare, comprising <3% of all infections amongst both blue and great tits). Note that this terminology reflects the identification of morphospecies that corresponded to distinct mtDNA lineages (Palinauskas et al. 2007), and updates previous work (Wood et al. 2007). Analyses using occupancy modelling have shown the qPCR assay technique is highly sensitive, with a very low probability of false negative diagnoses (Lachish et al. 2012); strict laboratory protocols ensure the likelihood of false positive diagnoses is small (Knowles et al. 2011).

Statistical and Spatial Analyses

Spatial analyses were based on the GPS-derived coordinates of each nestbox in which hosts were captured (Wilkin et al. 2007). As breeding tits are territorial and forage in the immediate vicinity (within 75m) of the nest (Stauss et al. 2005), these coordinates give an accurate

description of individuals' location during and either side of the breeding season, when transmission is most likely (Cosgrove et al. 2008). All analyses were performed separately for *P. relictum* and *P. circumflexum* infections in the two host species. The few individuals with mixed infections were considered twice in analyses (i.e. included in the spatial analyses conducted for both *Plasmodium* species); there were insufficient mixed infections for analysis of this class alone.

Kernel-smoothed relative risk maps

To characterise heterogeneity in infection risk of the two *Plasmodium* species across the study site, kernel-smoothed relative risk maps were produced to quantify the spatial variation in the odds of infection over time, using functions available in the 'sparr' package in R 2.12.1 (http://www.R-project.org). Kernel density estimation utilises a moving three-dimensional function with a specific search radius (bandwidth) to give a weighted mean density of point locations across the region of interest. For each year of the study, Gaussian edge-corrected kernel density estimation was performed on the locations of 'cases' (infected individuals) and 'controls' (uninfected individuals), with the ratio of the two density surfaces providing a relief map of the relative risk (odds) of infection across the study site, controlling for the underlying heterogeneity in the population at risk (Davies and Hazelton 2010, Kelsall and Diggle 1995). Optimum fixed bandwidths were obtained using the maximal smoothing principle of Terrell (1990) as recommended by (Davies and Hazelton 2010), with the same bandwidth used for the numerator and the denominator (Kelsall and Diggle 1995). Probability contours showing areas of significantly elevated risk were constructed based on the asymptotic properties of the fixed-bandwidth estimators and values obtained from the log-risk function (log of the ratio of the two surfaces; Davies and Hazelton 2010).

Local disease clusters

In parallel, the Kuldorff spatial scan statistic implemented in SaTScan™ (http://satscan.org/) was used to test for the presence of clusters of high *Plasmodium* infection rates within the study site and to identify their approximate locations and sizes (Kulldorff and Nagarwalla 1995). The spatial scan statistic creates a series of circular windows of variable radii around every infected individual, each of which is set to contain from zero to a defined maximum proportion of the total population-at-risk (the sum of cases and controls), which was here set to 25%. For each window, the observed number of cases is compared to the expected number of cases (given by a Bernoulli model and an assumption of constant risk). A maximum likelihood ratio test is then conducted to test the hypothesis that there is an elevated rate of infection within the window, when compared with the distribution outside, hence adjusting for heterogeneity in the background population-at-risk (with significance determined by simulated p-values using Monte Carlo methods with 999 replications; see Kulldorff and Nagarwalla 1995 for details).

Spatial variation in risk amongst paired individuals

We assessed whether observed spatial differences in infection risk with the two *Plasmodium* species (see Results) were also evident at the smallest spatial scale in our study, between paired birds, which are expected to share more similar environments than any other pair of individuals. Cuzick and Edwards' k-nearest neighbour test determines whether within a neighbourhood of 'k' neighbours of a case there are more likely to be other cases than other controls, (Cuzick and Edwards 1990). We applied the Cuzick and Edwards test to a dataset consisting only of breeding pairs where infection status was known for both pair members, and set 'k' (the number of nearest neighbours) to 1. Thus we directly tested whether the partner (1st nearest neighbour) of an infected individual was more likely to be a case than a

control. The test was performed using ClusterSeer 2 software (TerraSeer), with significance assessed using 999 Monte Carlo simulations, to randomise the case-control labels for each of the spatial locations and allow a comparison of the observed number of nearest-neighbour case pairs (T_k) to the distribution of T_k based on a random distribution of the data.

Environmental and host factors associated with disease clusters

Risk factor analysis for cluster membership (within a cluster or not) was undertaken in two stages and was based on results of the SatScan analysis, which identifies clusters of high infection rates. Analyses were restricted to *P. circumflexum* infections, as a significant cluster of *P. relictum* infection was only identified in one year of the study (see Results). We first employed multivariate logistic-regression models to assess the role of environmental factors and host density in determining the cluster membership of nestboxes occupied by blue tits or great tits. For each nestbox the following environmental predictors were obtained: (i) distance to the River Thames ('river'; the only large permanent water body within the site), (ii) distance to the woodland edge ('edge'), (iii) 'altitude', and (iv) number of oak trees within 50m ('oak'; a measure of local host food availability and territory quality). In addition, we obtained indices of the local density of blue tits ('BTdensity') and great tits ('GTdensity') by calculating the number of occupied nestboxes of each host species within 500m of each nestbox (qualitatively similar results were obtained using 100m and 250m radiuses). Indices were determined annually from nesting records rather than capture records, as not all parents of active nests were captured in each year. All explanatory variables were mean-centred before inclusion in models. Colinearity among explanatory variables was low: pair-wise correlations between variables were not extreme (Pearson's R^2 values ranged from 0.01 to 0.53, see Supplementary Table S1a) and variance inflation factors for all variables were low (<3.4, see Supplementary Table S1b). Hence, all variables were included in starting models,

along with relevant two-way interactions (river*oak, river*BTdensity, river*GTdensity). As the locations of *P. circumflexum* clusters overlapped significantly across years (see Results), we combined data from all years (such that each nestbox appeared once, was designated as within a cluster if observed as such at least once, and assigned an average local density calculated from the host-specific yearly values).

Akaike's information criterion, AIC, was used to determine the combination of variables that best explained the data with minimal parameters. Terms were sequentially removed from the full starting model and the fit of each new model assessed by comparison of AIC values. A decrease in AIC of >2 indicated an improved fit for the reduced model. When models differed in AIC by <2, the model with fewest parameters (terms) was considered the most parsimonious model (Burnham and Anderson 2002). To account for possible non-linear relationships with risk factors we then assessed whether the addition of quadratic functions of each remaining covariate improved the model fit (AIC). To confirm the validity of the minimum adequate model, eliminated variables were returned to the final model individually to assess any potential improvement in the fit of the final model.

The presence of residual spatial autocorrelation in the data was assessed through visual appraisal of binned semivariograms of the model residuals, against a simulation envelope constructed from 999 Monte Carlo permutations of the data, produced using the 'geoR' R package. Where significant spatial dependence in the residuals was identified (see Results), we re-ran the final minimum adequate models with an exponential spatial covariance structure using the *glmmPQL* function in the R package 'MASS' according to methods described in (Dormann et al. 2007). The presence of residual spatial autocorrelation in these

'spatial' models was again assessed by visual appraisal of semivariograms, as described above.

We next employed mixed-effects logistic models to assess the role of host-specific factors in predicting the cluster status of infected blue and great tits in each year of the study (the response variable being whether an infected individual was a 'cluster case' or not). Models were performed using the R package 'lme4' on data from all years of the study, with 'individual' included as a random effect to control for repeated captures of some individuals in the dataset. Starting models included the following fixed effects: (i) 'sex' (determined based on the presence or absence of a brood patch), (ii) 'age' (yearling or adult; determined from plumage characteristics or from ringing records), (iii) immigrant/resident status ('status'; which differentiated locally born individuals from those born elsewhere), and (iv) parasitaemia ('para'; the log-transformed density of parasites within infected hosts, measured via qPCR assay, as described in Knowles et al. 2011). An interaction between age and sex and a quadratic effect of parasitaemia were also included in starting models. Model selection proceeded as outlined above. Preliminary results, however, revealed the presence of significant residual spatial autocorrelation, which persisted even after the inclusion of an explicit spatial correlation structure in the model. Hence, to account for some of this unexplained spatial variation it was necessary to include distance to the River Thames ('river') as an additional fixed effect in all starting models.

RESULTS

An average of 467 (SD 87.51) blue tits were tested for *Plasmodium* infections each year from 2005 to 2010, with 645 and 471 great tits diagnosed in 2008 and 2009 respectively (see Table S2, Supplementary Material). Prevalence varied among years and between *Plasmodium*

species, with higher prevalence recorded for both *Plasmodium* species in great tits (Table S2).

Kernel-smoothed relative risk maps

Figure 1 shows the spatial variation in the relative risk (odds) of infection in each year for both *Plasmodium* species, scaled to the maximum and minimum values observed across all years for both host species, with arrows indicating the overall odds of infection in each year (i.e. number of cases/number of controls for each *Plasmodium* species, in each host species). These maps reveal striking differences in the spatial variation of the relative risk of *P. relictum* and *P. circumflexum* infection throughout the study site, with up to five-fold variation in infection risk across the woodland for the latter species (Figure 1).

The relative risk of *P. relictum* infection was broadly uniform across the study site, displaying little spatial variation in any year, though areas of elevated risk were occasionally observed (in 2006 and 2009 in blue tits, and in 2008 and 2009 for great tits; Figure 1). Aside from one small area (in 2006), these regions of elevated *P. relictum* risk were only of moderate significance ($P>0.01$), and were not always congruent in both host species (there was overlap in the area of elevated *P. relictum* risk for blue and great tits in 2009, but not in 2008; Figure 1). There was little difference in the maximum odds of *P. relictum* infection for blue and great tits, in years where comparison was possible (Figure 1).

In contrast, we observed distinct spatial heterogeneity in the relative risk of *P. circumflexum* infection across the study site, with a conspicuous north-south gradient of infection risk revealed in every year and for both host species (Figure 1). The risk of *P. circumflexum* infection was significantly elevated in the northern part of the study site ($P<0.01$, up to 5.5

times greater odds of infection), adjacent to the River Thames, the only large permanent water source near our study site (Figure 1). The risk of *P. circumflexum* infection in this northern area was higher for great tits than for blue tits, in both years where comparison was possible (Figure 1). However, the location and spatial extent of these elevated risk areas were very similar in all years of the study, and for both host species (geographic overlap among years for both host species ranged from 60% to 96% for the highest risk areas ($P<0.01$), and 78% to 97% for all areas of elevated risk ($P<0.05$); see Figure 2).

Location and size of local disease clusters

A significant cluster of *P. relictum* infections was identified in only one year of the study, 2006, indicating that, in general, the distribution of *P. relictum* cases is not very different from a random distribution in space (Figure 1). This single cluster encompassed a relatively large geographic area, and included the area identified as high relative risk of *P. relictum* infection, but was only of moderate significance ($P = 0.042$, Table S3 Supplementary Material). Clusters of *P. relictum* infection were not identified in areas of elevated *P. relictum* risk in other years (2008, 2009). Again, in contrast to the spatial pattern observed for *P. relictum*, highly significant ($P<0.001$) clusters of *P. circumflexum* cases were identified in every year of the study, and for both host species (Figure 1, Table S3). These disease clusters were consistently located in areas proximate to the River Thames, and within areas designated as high relative risk for *P. circumflexum* infection (Figure 2).

Spatial variation in risk amongst paired individuals

The results of the Cuzick and Edwards' k-nearest neighbour test also revealed marked differences between *P. relictum* and *P. circumflexum* in the risk of infection at the smallest spatial scale possible (between paired individuals), consistent with the broader scale patterns

observed across the study site (Table 1). For both blue and great tits, the partners of individuals infected with *P. circumflexum* were significantly more likely to be infected themselves (Table 1, with the exception of 2005). In contrast, partners of individuals infected with *P. relictum* were not more likely to be infected themselves, again indicating that the process of *P. relictum* infection is essentially random, even at this scale (Table 1). An exception to this pattern occurred in a single year of the study (2008) for both blue and great tits; however, the effect size was small compared to that for *P. circumflexum* (Table 1).

Environmental and host factors associated with disease clusters

Results of our analyses revealed similarities, but also some differences, in the factors that predicted the probability of *P. circumflexum* cluster membership for nestboxes occupied by blue tits and great tits (Tables 2 & 3). As residual spatial autocorrelation was present in both minimum adequate models identified (see Figure S1, Supplementary Material), final models (on which inference was based, see Tables 2 & 3) incorporated an explicit spatial covariance structure in residuals (for which no residual spatial autocorrelation was observed, Figure S1). Distance to the River Thames was by far the strongest predictor of the probability that a nestbox was part of a *P. circumflexum* cluster for both host species, with models supporting a linear relationship (on the logit scale) with this variable (Tables 2 & 3). Within 500m of the river virtually all nestboxes belonged to *P. circumflexum* clusters, whereas beyond 1200m of the river no nestbox belonged to a cluster (Figure 3 & Figure S2, Supplementary Material). Nestboxes further from the woodland edge were also more likely to be part of a *P. circumflexum* cluster for both host species (Tables 2 & 3), though the effect of this factor was substantially weaker and only apparent at intermediate distances from the river (Figures 3a & Figure S2a). Other environmental factors (altitude and oak abundance) had significant, but

substantially weaker, effects on the probability of nestbox cluster membership, which differed for blue tit and great tits (Tables 2 & 3, Figures 3b & Figure S2b).

An important finding revealed by these analyses was the differential influence of local host densities on the probability of cluster membership for nestboxes occupied by blue and great tits, revealing the importance of multi-host population dynamics in this disease system (Tables 2 & 3). The probability of belonging to a *P. circumflexum* cluster for nestboxes occupied by blue tits increased with the local density of great tits, but was not affected by the local density of blue tits (Table 2; Figure 3c). In contrast, for nestboxes occupied by great tits, the probability of cluster membership increased with the local density of both great tits and blue tits (Table 3, Figure S2c & S2d), though the influence of the latter diminished with distance from the river (Table 3, Figure S2c & S2d). These results did not appear to be a consequence of any systematic spatial arrangement of the two species, nor of any consistent difference between the abundance of these species over the study period (the density of great tits was higher than blue tits in 2008, but lower in 2009).

None of the host-specific risk factors examined strongly influenced the probability that an infected great tit belonged to a *P. circumflexum* cluster, indicating that great tit 'cluster cases' were not comprised of different types or classes of individuals (Table 4, with inference based on a model incorporating spatial covariance in residuals, Figure S3). In the blue tit analysis, however, model selection revealed that host parasite load (parasitaemia) influenced the probability that an infected individual was part of a *P. circumflexum* cluster (Table 4, with inference based on the minimum adequate model in which no residual spatial autocorrelation was identified, Figure S3). Infected blue tits within disease clusters tended to have higher parasite loads than infected blue tits elsewhere, though the relationship with this variable was

non-linear (on the logit scale), and the confidence intervals were wide (Figure S4, Supplementary Material).

DISCUSSION

Spatially explicit analyses of long-term, geo-referenced data on individual infection status in wild populations offer a powerful means of investigating the factors that shape spatial variation in infection risk with implications for epidemiology and host-parasite evolution (Ostfeld et al. 2005). We used this approach on a long-term dataset of avian malaria infections in two sympatric host species, infected with two *Plasmodium* parasites. We found pronounced parasite species-specific variation in the spatial patterns of disease risk, which is mirrored in two sympatric host species, and consistent between years.

Epidemiological insights into *P. circumflexum* transmission

Our analyses suggest that very different factors governing transmission of two closely related parasite species. Proximity to the River Thames (the only permanent body of water near our study site) is a key determinant of *P. circumflexum* infection risk not only for blue tits (Knowles et al. 2011, Wood et al. 2007; Lachish et al. 2011b), but also for a second sympatric host species, the great tit. For both host species, in each year, we observed a clear decline in the odds of *P. circumflexum* infection with increasing distance from the river. In addition, we showed that paired individuals experience a similar risk of *P. circumflexum* infection, indicating that transmission of this parasite is linked to the immediate local environment. In combination, these results suggest that differential exposure to vectors over a scale of several hundred metres generates the observed spatial variation in risk of *P. circumflexum* throughout the study site. Numerous studies have shown that proximity to water can restrict the distribution of mosquito vectors that are reliant on wet larval habitat for

breeding (Balls et al. 2004, Foley et al. 2003, Pope et al. 2005), with such effects also suggested for vectors of *P. circumflexum* in other systems (Krams et al. 2010). Knowledge of *Plasmodium* vectors in our study system is lacking, as is the case for most avian malaria studies, however, studies aimed at identifying the vectors of *Plasmodium* transmission within our study site are currently underway. The most abundant mosquito species in the area near the River Thames, *Ochlerotatus annulipes* (R. Alves unpublished data), lays its eggs exclusively on damp soil or leaf litter (Cranston et al. 1987) and is a potential candidate vector for *P. circumflexum*, as are *Culiseta annulata* and *Culiseta morsitans*, present within the study site (R. Alves, unpublished data) and known experimental vectors of *P. circumflexum* (Valkiūnas, 2005).

Although we found extensive overlap in the areas of elevated *P. circumflexum* risk for both host species, the odds of *P. circumflexum* infection within these areas were higher for great tits. As great tits are larger and are known to forage more widely than blue tits (Naef-Daenzer 1994), one explanation for this observation is that they attract, or encounter, vectors at higher rates than blue tits, thereby increasing their risk of infection. An alternative interesting possibility is that this result may be a consequence of differential impacts of *P. circumflexum* infection on host survival. Recently, we demonstrated that *P. circumflexum* infection entails significant survival costs for blue tits in this population (Lachish et al. 2011a). If great tits are not impacted to the same degree, then these species-specific differences in disease-induced mortality could manifest as higher odds of *P. circumflexum* infection for great tits.

Additional abiotic factors (distance from the woodland edge, altitude, and local oak abundance) were also associated with clustering of *P. circumflexum* infection, albeit to substantially lesser degree. These environmental variables may influence infection risk if

they further contribute to fine-scale spatial variation in conditions favourable for vector proliferation or vector competency. Alternatively, these environmental factors may influence spatial variation in *P. circumflexum* infection risk not just by limiting vector distributions, but by shaping host settlement patterns, and thus the fine-scale distribution of blood-meal hosts. Further work investigating both fine-scale variation in the distribution of mosquito species and host-specific settlement preferences is needed to shed light on these alternate possibilities.

An interesting finding of this study was that local host density, both conspecific and particularly heterospecific host density contributes to elevated risk of *P. circumflexum* infection, in areas close to the river (<1500m). Although we cannot rule out the possibility that vector densities were also elevated in these areas, our finding suggests that higher host densities promote more efficient transmission of *P. circumflexum* parasites (at least where vectors for transmission exist at sufficient abundance), an effect that has also been observed in other studies of avian blood parasites (Ortego and Cordero 2010). Transmission of vector-borne pathogens such as *Plasmodium* is typically characterised by frequency-dependence (the probability of infection is a function of the frequency, rather than the density, of infected hosts in a population), with transmission rates proportional to the vector/host ratio, so that transmission is expected to decrease at higher host densities (given a constant abundance of vectors, Antonovics et al. 1995). However, vector transmission rates are unlikely to be completely independent of population density (Thrall et al. 1995), and given the particular ecologies and behaviours of the hosts and vectors involved, a wider range of transmission dynamics along the continuum from frequency to density-dependence is likely for a variety of vector-borne pathogens (Antonovics et al. 1995). Further, a positive link between host density and infection rate could arise through indirect effects, if hosts at higher density are

subject to elevated stress with concomitant reduction in immunocompetence, spend less time on self-maintenance owing to high rates of interaction with conspecifics, or are of lower average quality than those at higher density (Morales et al. 2004).

However this link arises, it suggests that in this system host-specific transmission dynamics of *P. circumflexum* will be determined by the population dynamics of the entire host community. Thus, as noted for other multi-host pathogens (Craft et al. 2008, States et al. 2009), consideration of the community composition and densities of all alternate hosts will be necessary to explain the distribution and spatial variation in infection risk of this parasite species for a particular host. That clusters of *P. circumflexum* in blue tits were more likely in areas where the local density of great tits was high, but were not driven by conspecific host density, is intriguing and suggests that great tits may be more competent hosts for *P. circumflexum* infection, providing a local reservoir of infection for other susceptible hosts. In support of this suggestion, both *Plasmodium* prevalence and parasitaemia (parasite load in infected hosts) are higher in great tits than in blue tits at this study site (unpub data; see Table S2 for prevalence estimates, and Figure 1 for comparison of maximum odds of infection for the two species).

Our analyses revealed no evidence that infected individuals within clusters of *P. circumflexum* differed in age, sex, or site of birth (resident vs. immigrant), all factors that might reflect differences in host susceptibility to infection. However, we did find a weak but significant indication that for blue tits, clusters of *P. circumflexum* are comprised of hosts that harbour higher parasite loads. As spatial patterns of parasitaemia and prevalence in this population are independent (Knowles et al. 2011), this finding suggests there may be spatial variation in the ability of blue tits to suppress parasite *P. circumflexum* loads (immune

function) within the study site. Hence, we cannot entirely discount the possibility that spatial variability in host susceptibility to infection also contributes to spatial variation in *P. circumflexum* infection risk in this study.

Epidemiological insights into *P. relictum* transmission

In contrast to the distinct pattern of spatial variation in *P. circumflexum* infection risk observed across our study site, our analyses showed that for blue and great tits, infection with *P. relictum* was not much different from a random process (little consistent evidence of spatial clustering) both at an immediate local scale, between paired individuals, and over the whole study site. This result differs slightly from earlier findings, showing prevalence of *P. relictum* in blue tits at this site to be more southerly in distribution (Knowles et al. 2011, Wood et al. 2007). However, these previous studies lacked formal analysis of the spatial elements of infection and, more importantly, analysed data that was pooled over multiple years, which may have resulted in spatial structuring. Overall, the contrasting spatial patterns of infection risk for these two parasites suggests different vectors transmit them, with the possibility that the vector for *P. relictum* is less reliant on permanent wet larval habitats for breeding. Studies in other avian systems have shown that *Culex* species, in particular *Culex pipiens,* are important vectors of *P. relictum* (Valkiūnas, 2005). *Culex pipiens* (or its sibling species *Culex torrentium*) has been found at the study site breeding in temporary water bodies and rain-filled artificial water containers (R. Alves unpublished data), in accordance with its generalist breeding preferences (Cranston et al. 1987).

Since spatial structure of *Plasmodium* infections was defined in terms of host breeding site, an absence of strong spatial structuring of *P. relictum* infections might be seen if transmission occurs outside the host breeding season. Unlike *P. circumflexum*, which shows a distinct

post-breeding autumn peak in infections, the prevalence of *P. relictum* in this population displays no obvious seasonality (Cosgrove et al. 2008). Hence, transmission of *P. relictum* might occur over much longer time periods than *P. circumflexum* transmission, including times when birds are less strongly associated to a breeding site (although dispersal and settlement in the general area where breeding will take place tend to occur in the first autumn in both species). Investigating the timing of transmission for each of these parasites in relation to host dispersal will be a fruitful area for further research.

Evolutionary implications of spatial variation in *Plasmodium* infection risk for hosts

When parasites impose significant fitness costs for hosts, the presence of consistent spatial variation in infection risk will generate spatially variable parasite-mediated selection pressures that can drive local adaptation, and maintain genetic variation within the host population (Poulin 2007, Wolinska and King 2009). Previous work at this site has shown that *P. circumflexum* infection significantly reduces host survival (Lachish et al. 2011). Hence, the presence of such a pronounced spatial gradient in the risk of *P. circumflexum* infection across this study site, which was consistent in location over the six years studied here, implies the existence of spatially variable parasite-mediated selection pressures within this study site. Indeed, the temporal stability in spatial variation of *P. circumflexum* risk that we have demonstrated here is such that a degree of local adaptation is possible, providing there is genetic variation in the traits under selection (Templeton 2006).

A final implication of the pronounced spatial gradient in the risk of *P. circumflexum* infection revealed here is that host demography is expected to demonstrate parasite-driven spatial variation within our study site. If the observed survival cost of *P. circumflexum* infection (Lachish et al. 2011) constitutes additive mortality for hosts, then host mortality rates will

vary spatially, with, for example, substantially higher mortality in the area adjacent to the River Thames. A likely consequence of this is that other compensatory demographic processes, such as reproductive output, recruitment rates, or immigration rates will also vary spatially within this woodland site. Investigating the effect of such parasite-mediated, local-scale variation in these demographic processes on the dynamics of parasite transmission and on host meta-population processes will be a rewarding area for further research.

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Table 1: Results of the Cuzick and Edward's k-nearest neighbour test to assess the risk of infection amongst paired blue tits and great tits in each year of the study for *P. relictum* or *P. circumflexum*. The observed number of nearest-neighbour case pairs (T_k) is shown, along with the expected number based on random spatial distributions of cases and controls (E(T)), the variance (Var(T)), the test statistic (z) and the significance of the test statistic (P value) obtained from 999 Monte Carlo simulations (significant values denoted by *).

Plasmodium	**Host**	**Year**	**k**	**T(k)**	**E(T)**	**Var(T)**	**z**	**P value**
P. relictum	Blue Tits	2005	1	32	26.99	28.17	0.943	0.111
		2006	1	14	20.64	24.23	-1.348	0.987
		2007	1	42	51.51	43.20	-1.447	0.989
		2008	1	16	9.03	13.20	1.919	0.009*
		2009	1	4	8.37	12.35	-1.242	0.979
		2010	1	0	1.48	2.38	-0.959	1.000
	Great Tits	2008	1	38	24.63	30.68	2.413	0.001*
		2009	1	18	14.74	53.39	0.742	0.203
P. circumflexum	Blue Tits	2005	1	28	26.43	27.81	0.397	0.459
		2006	1	26	17.47	21.54	1.839	0.009*
		2007	1	22	13.14	17.81	2.098	0.004*
		2008	1	32	21.77	26.07	2.004	0.009*
		2009	1	44	32.05	33.40	2.067	0.004*
		2010	1	8	2.78	4.08	2.584	0.002*
	Great Tits	2008	1	89	58.47	53.39	4.177	0.001*
		2009	1	82	50.43	41.99	4.871	0.001*

Table 2: Results of model selection based on AIC for multivariate logistic models examining the influence of environmental risk factors and local species-specific host population density on the probability that nestboxes occupied by blue tits belong to a cluster of *P. circumflexum*. 'river' = distance to the River Thames, 'edge' = distance to the woodland edge, 'oak' = abundance of oak trees within 50m of a nestbox, 'BT density'/'GT density' = number of occupied nestboxes of each host within 500m of each nestbox. Test statistics reported are z, or t-statistics, for the non-spatial and spatial model respectively, with P-values for coefficients calculated from Wald tests. ΔAIC is the change in the AIC value after removal of that term from the model. Variables retained in the minimum adequate model are highlighted in bold

Model		**Covariate Effect**	**Coefficient**	**SE**	**Test statistic**	**P-value**	**ΔAIC**
Non-Spatial	Starting model	**river**	-0.0117	0.0013	-9.018	< 0.001	+463.73
		altitude	0.0281	0.0097	2.901	0.004	+6.41
		edge	0.0069	0.0023	3.005	0.003	+8.58
		GT density	0.0387	0.0202	3.396	< 0.001	+10.71
		oak	-0.0220	0.0188	-1.170	0.242	-0.63
		river*GT density	-0.0001	0.0001	-1.483	0.138	-0.02
		river*oak	-0.0001	0.0001	-1.056	0.291	-0.08
		BT density	0.0109	0.0121	0.898	0.369	-1.20
		river*BT density	0.0001	0.0001	0.363	0.717	-1.86
	Add quadratic terms	+river^2	-0.0000	0.0000	-0.387	0.699	+1.85
		+edge^2	-0.0000	0.0000	-0.147	0.882	+1.98
		+altitude^2	0.0006	0.0003	1.800	0.081	-0.99
		+oak^2	-0.0002	0.0010	-0.253	0.799	+1.94
Spatial	Add spatial covariance structure	**river**	-0.0117	0.0006	-18.759	< 0.001	
		altitude	0.0279	0.0047	6.038	< 0.001	
		edge	0.0070	0.0011	6.357	< 0.001	
		GT density	0.0677	0.0095	7.110	< 0.001	

Table 3: Results of model selection based on AIC for multivariate logistic models examining the influence of environmental risk factors and local species-specific host population density on the probability that nestboxes occupied by great tits belong to a cluster of *P. circumflexum*. 'river' = distance to the River Thames, 'edge' = distance to the woodland edge, 'oak' = abundance of oak trees within 50m of a nestbox, 'BT density'/'GT density' = number of occupied nestboxes of each host within 500m of each nestbox. Test statistics reported are z, or t-statistics, for the non-spatial and spatial model respectively, with P-values for coefficients calculated from Wald tests. ΔAIC is the change in the AIC value after removal of that term from the model. Variables retained in the minimum adequate model are highlighted in bold. Values indicated by * are the change in AIC for removal of all instances of that term from the model.

Model		**Covariate Effect**	**Coefficient**	**SE**	**Test statistic**	**P-value**	**ΔAIC**
Non-Spatial	Starting model	**river**	-0.0186	0.0038	-4.848	<0.001	+335.30*
		edge	0.0095	0.0042	2.270	0.023	+4.02
		oak	0.0990	0.0672	1.472	0.141	+10.68*
		BT density	-0.0175	0.0377	-0.463	0.643	+61.46*
		GT density	0.1946	0.0517	3.767	<0.001	+25.73
		river*oak	0.0004	0.0001	2.940	0.003	+7.08
		river*BT density	-0.0004	0.0001	-3.684	<0.001	+23.84
		river*GT density	0.0002	0.0001	1.767	0.081	+0.99
		altitude	-0.0332	0.0261	-1.270	0.204	-0.34
	Add quadratic terms	+river^2	0.0000	0.0000	1.549	0.121	-0.20
		+edge^2	0.0000	0.0001	0.856	0.392	+1.21
		+oak^2	0.0020	0.0017	1.152	0.249	+0.68
		+GT density^2	-0.0002	0.0018	-0.098	0.922	+1.02
		+BT density^2	0.0013	0.0010	1.257	0.209	-0.10
Spatial	Add spatial covariance structure	**river**	-0.0106	0.0010	-11.758	<0.001	
		edge	0.0062	0.0015	4.198	<0.001	
		oak	0.0445	0.0132	3.374	<0.001	
		BT density	0.0228	0.0093	2.439	0.015	
		GT density	0.0611	0.0073	8.360	<0.001	
		river*oak	0.0001	0.0001	5.216	<0.001	
		river*BT density	-0.0001	0.0001	-5.235	<0.001	

Table 4: Results of model selection based on AIC for multivariate logistic models examining the influence of host factors on the probability that infected individuals belong to a cluster of *P. circumflexum*. 'river' = distance to the River Thames, 'para' = parasitaemia, or host parasite load. Test statistics reported are z, or t-statistics, for the non-spatial and spatial model respectively, with P-values for coefficients calculated from Wald tests (†values reported for Great Tits are from the final spatial model). ΔAIC is the change in the AIC after removal of that term from the model. Variables retained in the minimum adequate model are highlighted in bold. Values indicated by * are the change in AIC for removal of all instances of that term from the model.

Dataset	**Host Species**	**Covariate Effect**	**Coefficient**	**SE**	**Test statistic**	**P-value**	**ΔAIC**
Infected individuals only	Blue Tits	**river**	-0.012	0.001	-9.674	< 0.001	+595.51
		para	0.407	0.263	1.546	0.122	+14.12*
		para^2	-0.092	0.034	-2.676	0.007	+5.81
		status	0.587	0.402	1.462	0.144	-0.20
		sex	0.469	0.370	1.268	0.205	-0.40
		age	0.013	0.369	0.034	0.973	-2.00
		age*sex	0.254	0.743	0.342	0.732	-1.90
	Great Tits†	**river**	-0.017	0.003	-6.061	<0.001	+319.50
		para	0.187	0.203	0.921	0.357	-0.40
		para^2	0.038	0.074	0.520	0.603	-4.51
		status	-0.727	1.460	-0.498	0.619	-0.82
		sex	1.097	1.500	0.732	0.464	-0.60
		age	0.228	1.476	0.155	0.877	-3.90
		age*sex	-0.255	2.926	-0.087	0.930	-1.30

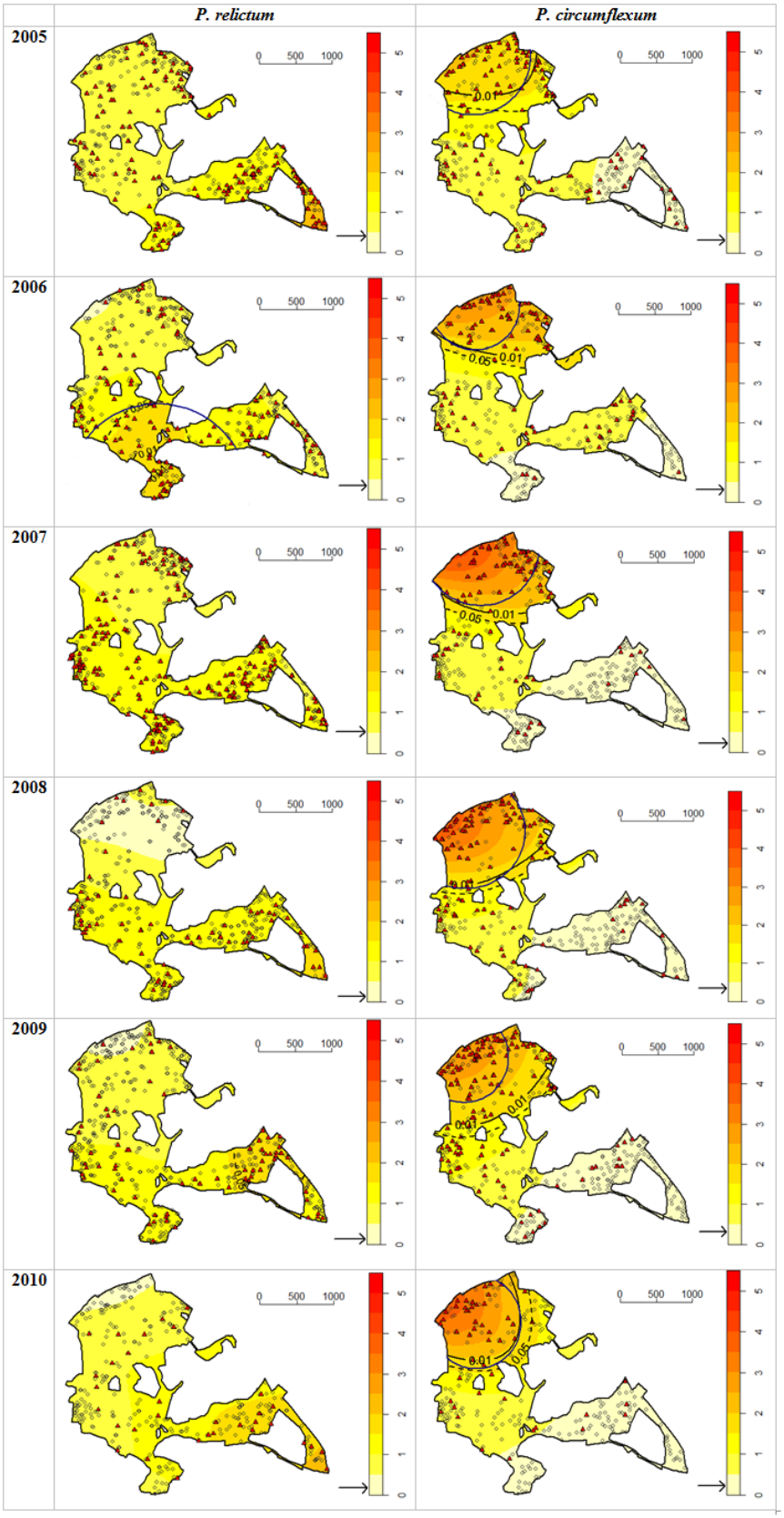
Figure 1: Kernel-smoothed maps showing relative risk of infection with *P. relictum* or *P. circumflexum* in each year of the study, across the study site for (a) blue tits and (b) great tits. Value ranges (depicted by colours) have been scaled to the maximum and minimum values observed across all years for both host species. Contour lines depict areas of significantly elevated relative risk (solid line for $P<0.01$; dashed line for $P<0.05$). The locations of 'cases' in each year are shown by red filled triangles; those of 'controls' by open circles. Arrows indicate the overall odds of infection in each year (i.e. total number of cases/total number of controls). In addition, the location and spatial extent of statistically significant clusters of cases (as determined by SatScan analyses), are shown by blue circles.

Figure 2: Maps overlaying the areas identified as high relative risk of *P. circumflexum* infection ($P<0.05$) for (a) blue tits and (b) great tits. The grey circles on these maps show the locations of all available nestboxes within the study site, while the hatched line shows the location of the River Thames.

Figure 3: Probability of cluster membership for nestboxes occupied by blue tits as a function of distance from the River Thames and (a) distance from the woodland edge, (b) altitude and (c) the local density of great tits. Predictions were generated from the minimum adequate spatial model (see Methods). Solid lines indicate the mean response in all cases; dotted lines show predicted values at the minimum value of the covariate; dashed lines show predicted values at the maximum value of the covariate. All other terms in the model were set to their mean value. Circles are the observed values.

Figure 1

(a)


P. relictum
P. circumflexum
2005
2006
2007
2008
2009
2010
0 500 1000
0 1 2 3 4 5
0.01
0.05
0.01

(b)

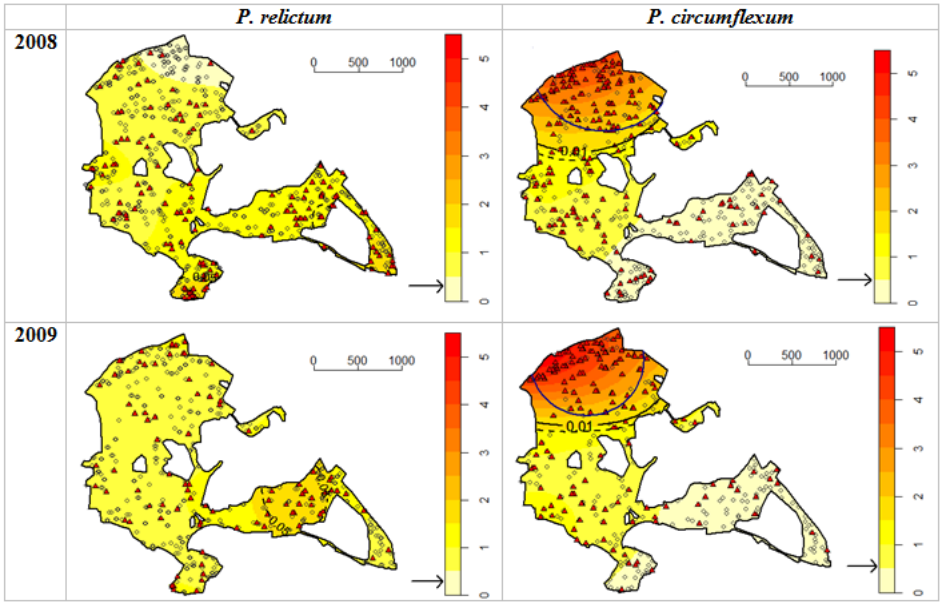
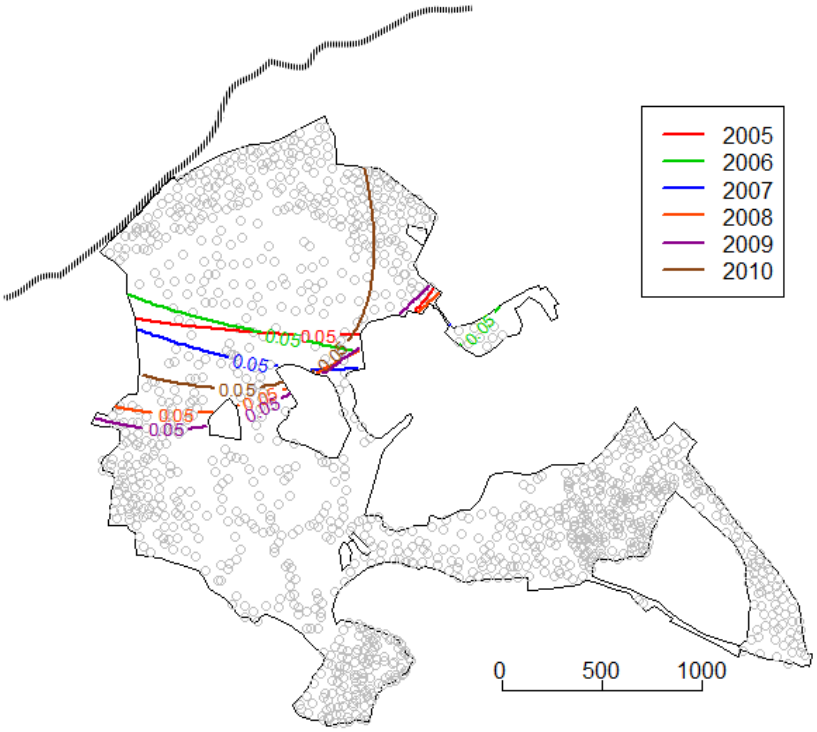

P. relictum
P. circumflexum
2008
2009
0 500 1000
0 1 2 3 4 5
0.01
0.05

Figure 2

(a)


2005
2006
2007
2008
2009
2010
0.05
0.05
0.05
0.05
0.05
0.05
0.05
0.05
0.05
0
500
1000

(b)

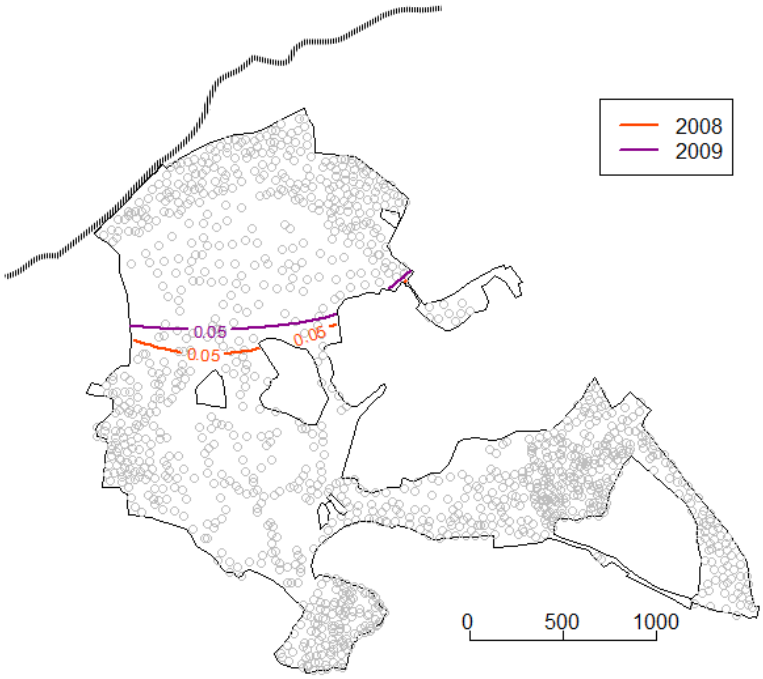
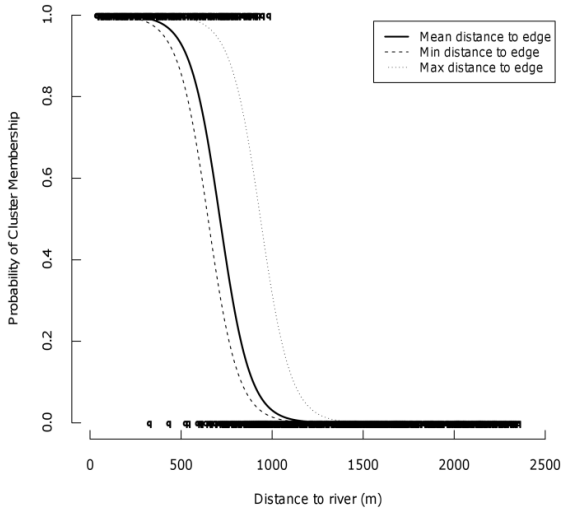
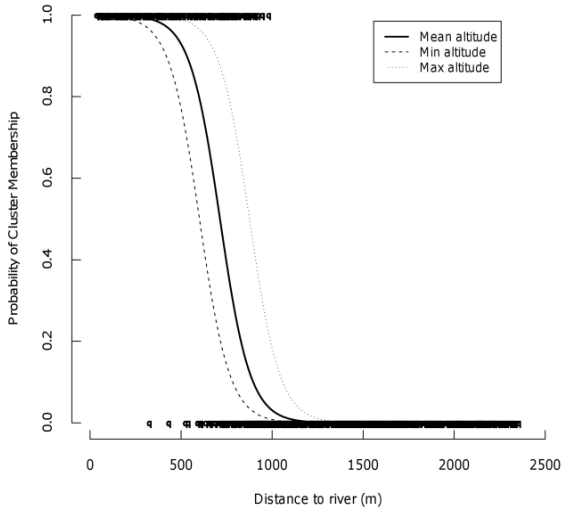

2008
2009
0.05
0.05
0.05
0
500
1000

Figure 3

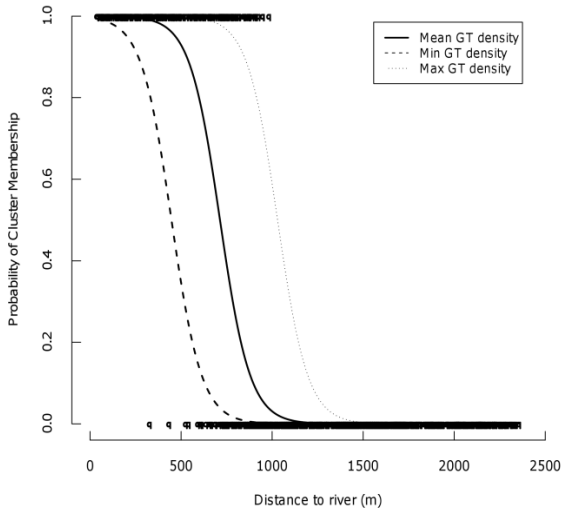
(a)


Mean distance to edge
Min distance to edge
Max distance to edge
Probability of Cluster Membership
0.0
0.2
0.4
0.6
0.8
1.0
0
500
1000
1500
2000
2500
Distance to river (m)

(b)


Mean altitude
Min altitude
Max altitude
Probability of Cluster Membership
0.0
0.2
0.4
0.6
0.8
1.0
0
500
1000
1500
2000
2500
Distance to river (m)

(c)


Mean GT density
Min GT density
Max GT density
Probability of Cluster Membership
0.0
0.2
0.4
0.6
0.8
1.0
0
500
1000
1500
2000
2500
Distance to river (m)

Supplementary Information

Table S1: (a) Pair-wise correlations and (b) variance inflation factors of the six explanatory variables used in generalised linear models performed for blue tits and great tits†, investigating environmental and host factors associated with disease cluster membership of nestboxes.

(a)

	river	altitude	edge	oak	BTdensity	GTdensity
river	--	0.423	-0.279	0.009	-0.335	-0.056
altitude	0.338	--	0.213	-0.134	0.108	0.016
edge	-0.331	0.182	--	-0.042	0.415	0.281
oak	-0.162	-0.107	0.038	--	-0.153	-0.156
BTdensity	-0.161	0.011	0.441	0.032	--	0.528
GTdensity	-0.249	-0.164	0.199	0.053	0.461	--

† Correlations for blue tits are shown above the diagonal, and for great tits below the diagonal

(b)

	Blue tit model	Great tit model
river	1.895	3.293
altitude	2.545	2.607
edge	1.414	2.452
oak	1.274	1.318
BT density	1.645	1.702
GT density	1.696	2.522

Table S2: Summary table showing the number of blue tits and great tits captured and diagnosed with either *P. relictum* or *P. circumflexum* infections in Wytham Woods in each year of the study, along with the prevalence of each pathogen in the population.

Plasmodium	**Host**	**Year**	**# Infected**	**Total**	**Prevalence**
P. relictum	Blue Tit	2005	138	467	0.296
		2006	115	471	0.244
		2007	183	522	0.351
		2008	70	517	0.135
		2009	75	529	0.142
		2010	34	297	0.114
	Great Tit	2008	134	645	0.208
		2009	90	471	0.191
P. circumflexum	Blue Tit	2005	123	467	0.263
		2006	102	471	0.217
		2007	92	522	0.176
		2008	114	517	0.221
		2009	137	529	0.259
		2010	52	297	0.175
	Great Tit	2008	214	645	0.332
		2009	167	471	0.355

Table S3: Summary results of SatScan cluster analysis showing the observed and expected number of cases within each cluster (O/E), the log-likelihood ratio (LLR), and the significance (P value) of each of the identified SatScan clusters (clusters locations and sizes shown in Figure 1).

Plasmodium	**Host**	**Year**	**LLR**	**P value**	**O/E**
P. relictum	Blue Tit	2006	8.082	0.042	45/28.32
P. circumflexum	Blue Tit	2005	13.101	<0.0001	52/30.29
		2006	22.025	<0.0001	42/17.76
		2007	34.674	<0.0001	51/19.03
		2008	32.238	<0.0001	60/26.24
		2009	29.337	<0.0001	60/27.45
		2010	21.826	<0.0001	32/12.26
	Great Tit	2008	50.371	<0.0001	106/53.90
		2009	50.480	<0.0001	87/41.48

Figure S1: Binned semivariograms of the standardised residuals of (a) the minimum adequate models identified by model selection for the multivariate logistic regression models assessing the influence of environmental/host density factors on the probability of nestbox cluster membership (see Methods), and (b) the same models with an explicit exponential spatial covariance structure in the residuals included. Dashed lines represent the simulation envelope based on 999 Monte Carlo permutations of the data.

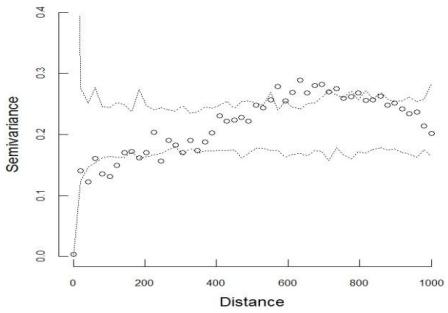
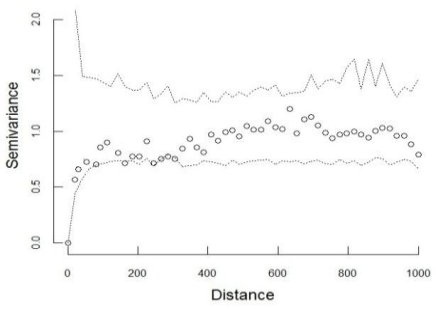
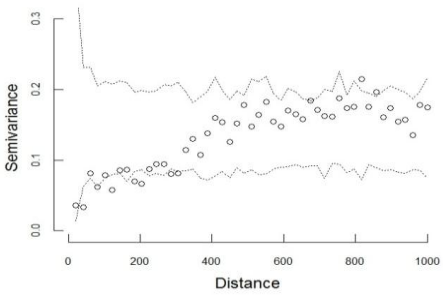
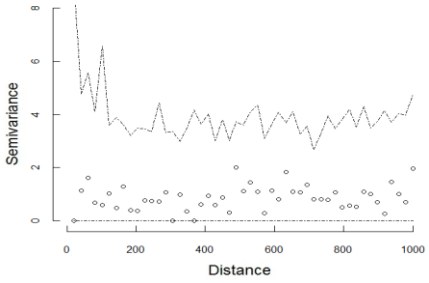
Host Species	(a) Minimum adequate models (non-spatial)	(b) Spatial model (spatial covariance structure in residuals)
Blue Tits		
Great Tits		

Figure S2: Probability of cluster membership for nestboxes occupied by great tits as a function of distance from the River Thames and (a) distance from the woodland edge, (b) oak abundance and (c) the local density of great tits and (d) the local density of blue tits. Predictions were generated from the minimum adequate spatial model (see Methods). Solid lines indicate the mean response in all cases; dotted lines show predicted values at the minimum value of the covariate; dashed lines show predicted values at the maximum value of the covariate. All other terms in the model were set to their mean value. Circles are the observed values.

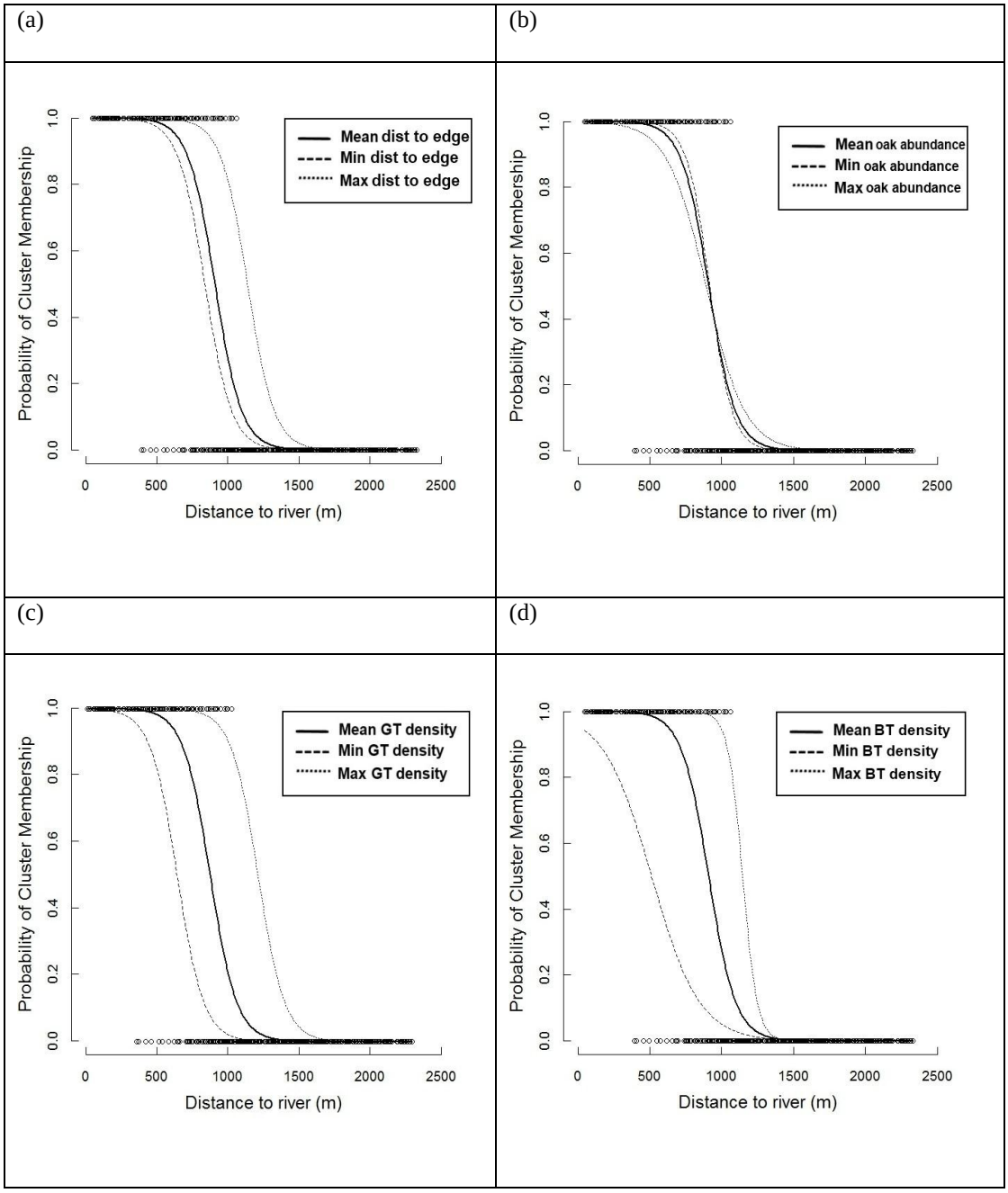


Figure S3: Binned semivariograms of the residuals of the minimum adequate models identified by model selection for the mixed-effects logistic models assessing the influence of host-specific factors on the probability of *P. circumflexum* cluster membership for infected blue tits and great tits (see Methods). Dashed lines represent the simulation envelope based on 999 Monte Carlo permutations of the data.

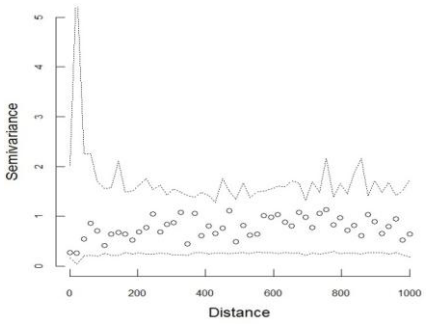
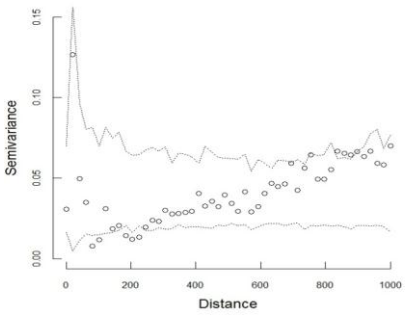
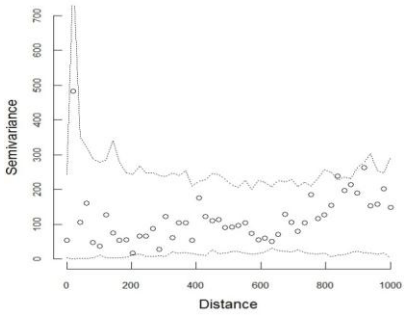
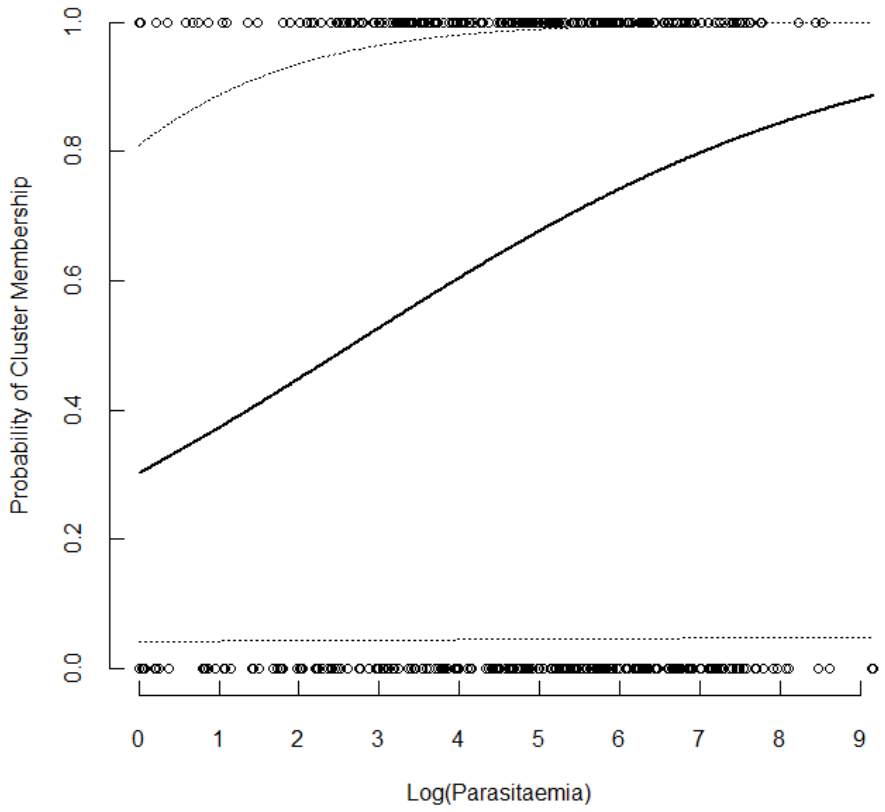
Host Species	(a) Minimum adequate models (non-spatial)	(b) Spatial model (spatial covariance structure in residuals)
Blue Tits		Not required
Great Tits		

Figure S4: Probability of *P. circumflexum* cluster membership for blue tits infected with *P. circumflexum* as a function of parasitaemia. Predictions were generated from the minimum adequate model (see Methods). Dotted lines are 95% CI. Circles are the observed values.



Example of plotted deviance residuals against fitted values for a GLM model fitted alternately with different error structures

Plotted deviance residuals against fitted values for the full GLM constructed to explore species-specific associations of *An. plumbeus* abundance with six environmental variables (Chapter 3). The GLM was fitted alternately with three different error structures: negative binomial, quasi-Poisson and Gaussian (using log transformed dependent variables). Choice of the most appropriate error structure (Gaussian) was based on absence of trends in the spread of plotted residuals (Zuur *et al*., 2009).