Plasmodium transmission differs between mosquito species and parasite lineages

Rafael Gutiérrez-López[1,*] , Josué Martínez-de la Puente[1,2] ,

Laura Gangoso[1,3], Ramón Soriguer[2,4] and Jordi Figuerola[1,2]

[1]Department of Wetland Ecology, Estación Biológica de Doñana (EBD-CSIC), Calle Américo Vespucio 26, E-41092 Seville, Spain; [2]CIBER Epidemiología y Salud Pública (CIBERESP), Seville, Spain; [3]Theoretical and Computational Ecology, Institute for Biodiversity and Ecosystem Dynamics, University of Amsterdam, Amsterdam, The Netherlands and [4]Department of Ethology, Estación Biológica de Doñana (EBD-CSIC), Calle Américo Vespucio 26, E-41092 Seville, Spain

Abstract

Factors such as the particular combination of parasite–mosquito species, their co-evolutionary history and the host's parasite load greatly affect parasite transmission. However, the importance of these factors in the epidemiology of mosquito-borne parasites, such as avian malaria parasites, is largely unknown. Here, we assessed the competence of two mosquito species [*Culex pipiens* and *Aedes* (*Ochlerotatus*) *caspius*], for the transmission of four avian *Plasmodium* lineages (*Plasmodium relictum* SGS1 and GRW11 and *Plasmodium cathemerium*-related lineages COLL1 and PADOM01) naturally infecting wild house sparrows. We assessed the effects of parasite identity and parasite load on *Plasmodium* transmission risk through its effects on the transmission rate and mosquito survival. We found that *Cx. pipiens* was able to transmit the four *Plasmodium* lineages, while *Ae. caspius* was unable to transmit any of them. However, *Cx. pipiens* mosquitoes fed on birds infected by *P. relictum* showed a lower survival and transmission rate than those fed on birds infected by parasites related to *P. cathemerium*. Non-significant associations were found with the host–parasite load. Our results confirm the existence of inter- and intra-specific differences in the ability of *Plasmodium* lineages to develop in mosquito species and their effects on the survival of mosquitoes that result in important differences in the transmission risk of the different avian malaria parasite lineages studied.

Introduction

Parasites of the genus *Plasmodium*, the causative agent of malaria, are vector-borne Haemosporidians that greatly affect humans and wildlife (Sachs and Malaney, 2002; Valkiūnas, 2005). The avian malaria parasite *Plasmodium* shows a wide range of competent hosts belonging to different bird orders and families (Fallon *et al*., 2005; Pérez-Tris *et al*., 2007; Hellgren *et al*., 2009). These parasites are transmitted by some competent mosquito species, in which they undergo a sexual reproduction phase (Valkiūnas, 2005). Infective forms of the parasite migrate to the mosquito salivary glands and may be then transmitted to a new avian host. Consequently, mosquito–*Plasmodium* interactions may play an important role in the dynamics of parasite transmission (Kimura *et al*., 2010). As stated above, not all mosquito species are competent vectors of avian malaria parasites. The main vectors are mosquitoes of the genus *Culex*, although other genera such as *Aedes* or *Anopheles* could also be involved in their transmission (Santiago-Alarcon *et al*., 2012). The finding of genetically related parasite lineages/species in different mosquito genera leads to the assumption of a generalist relationship between *Plasmodium* and mosquitoes (Kimura *et al*., 2010; Ferraguti *et al*., 2013; Schoener *et al*., 2017). However, most information regarding the mosquito species involved in avian *Plasmodium* transmission is based on the molecular identification of parasite DNA on mosquito pools, without a quantitative evaluation of the vector competence for different mosquito species (Kimura *et al*., 2010; Ferraguti *et al*., 2013; Schoener *et al*., 2017). Indeed, molecular detection of parasite DNA in insects' bodies does not imply these are competent vectors (Valkiūnas, 2011) and interspecific differences in competence for the transmission of avian *Plasmodium* could be overlooked, as parasite DNA may also be isolated from the body of non-competent mosquito species (Beerntsen *et al*., 2000; Ishtiaq *et al*., 2008). Thus, it becomes crucial to evaluate the competence of different mosquito species for the transmission of different *Plasmodium* lineages to better understand the transmission of avian malaria parasites in the wild.

For the successful transmission of avian *Plasmodium*, vectors must survive long enough to allow parasites to complete their life cycle (between 8 and 13 days) (Valkiūnas, 2005; LaPointe *et al*., 2010). The development of *Plasmodium* in the mosquito may be affected by several environmental factors, such as temperature and humidity (Paaijmans *et al*., 2010; Lefévre *et al*., 2013). In addition, the particular species involved in the parasite–vector assemblage and the vertebrate host parasite load may further determine the success of development of parasites in mosquitoes (Cornet *et al*., 2014). For example, mosquitoes feeding on birds with high parasite loads develop a high density of ookinetes (an initial non-infective phase

of *Plasmodium*) in their abdomen, likely increasing parasite transmission success (Pigeault *et al.*, 2015). However, *Plasmodium* development in the mosquito produces tissue damage, with potential negative consequences for mosquito survival. Previous studies on the impact of avian malaria parasites on vector survival have reported positive, negative or non-significant effects of parasite infection on mosquito longevity (Vézilier *et al.*, 2012; Lalubin *et al.*, 2014; Delhaye *et al.*, 2016; Pigeault and Villa, 2018; Gutiérrez-López *et al.*, 2019*a*). However, most of these studies focus on the interaction between *Culex pipiens* mosquitoes and *Plasmodium relictum* (lineage SGS1) (Cornet *et al.*, 2013; Pigeault *et al.*, 2015; Martínez-de la Puente *et al.*, 2018). Therefore, studies considering potential differences in virulence (i.e. the cost of the pathogen infections on their host) between parasite species/lineages on different mosquito species are necessary (Lachish *et al.*, 2011).

Here, we experimentally assessed the competence of two mosquito species, *Cx. pipiens* and *Aedes* (*Ochlerotatus*) *caspius*, for the transmission of four avian *Plasmodium* lineages. Both mosquito species are common in southern Spain, where they show different feeding patterns. While *Cx. pipiens* feed mainly on birds (Martínez-de la Puente *et al.*, 2016), *Ae. caspius* prefers to bite mammals, although birds may represent up to 19% of their diet (Balenghien *et al.*, 2006; Muñoz *et al.*, 2012; Gutiérrez-López *et al.*, 2019*b*). Avian *Plasmodium* DNA has been isolated from both mosquito species (Ferraguti *et al.*, 2013; Schoener *et al.*, 2017), and the capacity of *Cx. pipiens* for the transmission of avian *Plasmodium* parasites has been previously demonstrated (Kazlauskiené *et al.*, 2013; Gutiérrez-López *et al.*, 2016; Palinauskas *et al.*, 2016). In this study, mosquitoes were allowed to feed on birds naturally infected by *Plasmodium* to assess the effects of bird parasite load and parasite identity (i.e. different *Plasmodium* lineages grouped into main clades, see below) on the probability of mosquito infection and parasite transmission. We also analysed the impact of parasite development on mosquito survival. Finally, we estimated the impact of mosquito survival and parasite development on the risk of parasite transmission, based on the quantification of the relative basic reproductive number (R_0), modified from Ross (1911) and Macdonald (1955).

Materials and methods

Mosquito collection and rearing

Larvae of *Cx. pipiens* and *Ae. caspius* were collected from April to September in 2014 and 2016 in the natural reserve 'La Cañada de los Pájaros' (6°14′W, 36°57′N, Seville Province, Spain) and in marshlands of the Huelva Province (6°53′W, 37°17′N). Larvae were grown in plastic trays with fresh or brackish water, respectively, maintained following Gutiérrez-López *et al.* (2019*a*) with food *ad libitum*. Adult female mosquitoes were identified to the species level following Schaffner *et al.* (2001), placed in insect cages (BugDorm-43030F, 32.5 × 32.5 × 32.5 cm) and fed *ad libitum* with 1% sugar solution. One day prior to each experiment, 2–3-week-old female mosquitoes were deprived from sugar solution. Only F0 generation mosquitoes collected in the field were used in the experiment. Laboratory maintained colonies of mosquitoes were not used to minimize the potential effects of artificial selection on vector–host interactions (Franks *et al.*, 2011; Lagisz *et al.*, 2011).

Bird sampling and experimental procedure

A total of 60 wild house sparrows (*Passer domesticus*) were caught from May to September 2014 in 'La Cañada de los Pájaros' and from June to September 2016 in different localities from Huelva Province using mist nets. Birds were individually ringed and weighed. Blood samples (0.2 mL) were obtained by jugular venepuncture using sterile syringes. A drop of blood was smeared, airdried, fixed with absolute methanol and stained with Giemsa for 45 min (Gering and Atkinson, 2004). The rest of the blood was transferred to non-heparinized Eppendorf tubes to perform molecular detection of parasites (see below). A total of 4000–10 000 erythrocytes from each smear were scanned at high magnification (×1000). *Plasmodium* parasite load was estimated as the percentage of infected erythrocytes. Although the gametocytaemia (proportion of red blood cells infected by gametocytes, i.e. the sexual stage of the parasite that is transmitted to mosquitoes) may provide a more reliable quantitative measure of parasite infection than parasitaemia, both variables are strongly correlated (Pigeault *et al.*, 2015).

Forty-five individual birds were enclosed for 30 min in an insect cage (BugDorm-43030F, 32.5 × 32.5 × 32.5 cm) containing either *Cx. pipiens* (mean ± S.D.: 68.48 ± 34.97, range 4–111) or *Ae. caspius* (57.29 ± 34.04, range 4–126) females. Although we tried to use a similar number of mosquitoes per box, this task was difficult to achieve due to the fact that mosquitoes were obtained from larvae in the field, which limited the number of mosquitoes of similar age/species available for each day/trial. Birds were immobilized to prevent defensive behaviours following Gutierrez-López *et al.* (2019*b*). The feeding trials were undertaken from 7:30 to 12:00 h (GMT + 1 h). Because Gutiérrez-López *et al.* (2019*b*) did not find any effect of house sparrow sex on *Cx. pipiens* and *Ae. caspius* biting rates, we grouped birds from both sexes in this study, including 18 females and 42 males. At the end of each trial, all birds were immediately released at the place of capture, with no apparent sign of damage. In addition, 15 of these 60 birds corresponded to control birds from the study of Yan *et al.* (2018). These birds were injected with saline solution, maintained in captivity for 24 days before being exposed to mosquitoes in the context of Yan *et al.* (2018) study. Twenty-four hours after this exposure, we re-exposed these 15 birds to mosquitoes in the context of this study following the same procedure described above.

After trials, engorged mosquitoes (see Table 1) were placed in insect cages, one for all mosquitoes fed on the same individual bird, and maintained under the same conditions detailed above. Mosquito survival was monitored every 12 h until 13 days postexposure (dpe). At the end of this period, the saliva of surviving mosquitoes was obtained following the protocol detailed by Gutiérrez-López *et al.* (2016). We chose the isolation of saliva over other conventional methods such as the analysis of mosquito salivary glands because the former allows the use of molecular methods for parasite detection and it has been widely used in studies on the competence of mosquitoes to transmit other pathogens such as viruses (Goddard *et al.*, 2002; Dubrulle *et al.*, 2009; Gutiérrez-López *et al.*, 2019*c*) and malarial parasites (Golenda *et al.*, 1992; Gutiérrez-López *et al.*, 2019*a*). Subsequently, the head-thorax of each mosquito, which contains the salivary glands, was separated from the abdomen in a sterile Petri dish. Samples were kept at −80°C until further molecular analyses.

Molecular analyses

Genomic DNA was extracted from bird blood samples and the head-thorax of mosquitoes using the MAXWELL® 16 LEV Blood DNA Kit while the Qiagen DNeasy® Kit Tissue and Blood (Qiagen, Hilden, Germany) was used to extract the DNA from mosquito saliva (Gutiérrez-López *et al.*, 2015). The presence of *Plasmodium*/*Haemoproteus* parasites was determined following Hellgren *et al.* (2004). Positive amplifications were sequenced in both directions using the BigDye technology (Applied Biosystems) or with the Macrogen Inc. (Amsterdam, The Netherlands) sequencing service. Sequences were edited using software Sequencher™ v4.9 (Gene

Table 1. *Cx. pipiens* and *Ae. caspius* engorged and analysed for the different *Plasmodium* lineages found in house sparrows

Mosquito species	*Plasmodium* lineages	Clade	*N* house sparrows	Engorged mosquitoes	Alive until 13 dpe	Head-thorax positive/analysed	Saliva positive/analysed
Cx. pipiens	*P. relictum* SGS1	A	15	112	63[a]	21/60	2/21
	P. relictum GRW11	A	4	35	33	14/33	2/14
	Plasmodium COLL1	B	6	23	20	8/20	2/8
	Plasmodium PADOM01	B	2	13	13	8/13	5/8
Ae. caspius	*P. relictum* SGS1	A	11	58	22	0/22	–
	P. relictum GRW11	A	2	30	14	0/14	–
	Plasmodium COLL1	B	2	17	8	0/8	–
	Plasmodium PADOM01	B	2	7	1	0/1	–

The number of individual house sparrows infected with each *Plasmodium* lineage is shown.
[a]Three mosquitoes fed on birds infected with *P. relictum* escaped.

Codes Corp., © 1991–2009, Ann Arbor, MI 48108, USA) and assigned to parasite lineages/morphospecies after comparison with public databases [GenBank and MalAvi (Bensch *et al.*, 2009; Kumar *et al.*, 2016)]. The four *Plasmodium* lineages found in this study were grouped into two main clades (clade A and clade B, see 'Results' section) and uncorrected *p*-distances between lineages/clades were compared using MEGA7 software (Kumar *et al.*, 2016). Five birds showed evidence of coinfection, as revealed by the existence of double peaks in the sequencing chromatogram, and were not included in this study to avoid potential confounding effects of multiple infections on parasite development and mosquito survival (Lover and Coker, 2015). Ten house sparrows uninfected by avian *Plasmodium* (see 'Results' section) were used as control to study the survival of 80 mosquitoes that fed on them.

Statistical analyses

Statistical analyses were performed in R software 3.2.5 (R Core Development Team, 2016). We fitted two similar generalized linear mixed models with binomial error and logit link function using the package *lme4* (Bates *et al.*, 2015), to assess the effects of *Plasmodium* clade identity (fixed factor) and the bird parasite load (covariate) on the status of infection by *Plasmodium* (infected/uninfected) in the head-thorax or saliva, respectively. The variable bird parasite load was log-transformed to attain normality. In both models, bird identity was included as a random term. We fitted, by maximum likelihood using the package *survival* (Therneau and Lumley, 2014), a mixed-effects Cox model to test the effects of bird parasite infection status (fixed factor, infected/uninfected birds) and parasite load (covariate) on mosquito survival (measured as the number of mosquitoes alive at each 12-h-period), and bird identity as a random factor, while controlling for the potential effect of mosquito age (2 or 3 weeks old). We also fitted a similar mixed-effects Cox model with parasite identity (clade A, clade B and uninfected) instead of infection status as explanatory variable. We restricted the analyses of survival to *Cx. pipiens* mosquitoes, as *Plasmodium* only developed successfully in this species (see 'Results' section).

Plasmodium transmission risk

We used a simplified equation of the R_0 model proposed by Macdonald (1955) to calculate relative R_0 values:

$$R_{0,\text{rel}} = \frac{c}{(-\ln P)} P^\nu$$

where *c* represents the probability of a mosquito becoming infected after biting an infected host, *P* is the daily survival rate of mosquitoes measured as the probability that a mosquito survives for 1 day and *ν* is the pathogen incubation period in the mosquito. In our study, *c* (hereafter, transmission rate) was considered as the probability of a mosquito carrying *Plasmodium* DNA in its saliva after feeding on an infected bird. In addition, we considered *ν* as 13 days (Valkiūnas, 2005; LaPointe *et al.*, 2010). The relative R_0 value was calculated considering the survival rate until 13 dpe and the proportion of mosquitoes with positive saliva samples infected with lineages of each *Plasmodium* clade. The relative R_0 provides an approach to quantify the impact of differences in the survival rate of mosquitoes or *Plasmodium* transmission to mosquito saliva on *Plasmodium* transmission.

Results

Avian malaria parasites in birds

From the 60 birds captured, 10 were uninfected (and the mosquitoes that fed on them were used as controls) and 50 were infected by at least one avian malaria lineage. Five of them showed coinfection by several lineages of haemosporidians and were removed from further analyses. Overall, we exposed 45 birds infected with four parasite lineages, including the *P. relictum* lineages SGS1 (*N* = 26) and GRW11 (*N* = 6), and the lineages COLL1 (*N* = 8) and PADOM01 (*N* = 5) (Table 1), to mosquitoes. The morphospecies for COLL1 and PADOM01 are unknown, but these lineages clustered with the lineage SEIAUR01, corresponding to *Plasmodium cathemerium*, which was already found in house sparrows in the same area (Ferraguti *et al.*, 2018) (Fig. 1). The uncorrected *p*-distance between lineages SGS1 and GRW1 and between COLL1 and PADOM01 was 0.002 (corresponding to a difference of a single base pair). By contrast, the uncorrected *p*-distance between lineages SGS1–GRW11 and COLL1–PADOM01 was 0.035. Thus, in further analyses these four lineages were grouped into two different clades: clade A corresponding to the *P. relictum* lineages SGS1 and GRW11, and clade B corresponding to the *Plasmodium* spp. lineages COLL1 and PADOM01, which are closely related to *P. cathemerium*. The parasite load in birds did not differ significantly between clades (mean ± S.D.: clade A: 1.39 ± 0.21, clade B: 1.15 ± 0.35, ANOVA; $F_{1,37} = 0.34$, $P = 0.56$). In addition, the parasite load was similar between parasite lineages within clade A (ANOVA; $F_{1,27} = 0.03$, $P = 0.87$) and clade B (ANOVA; $F_{1,8} = 0.06$, $P = 0.81$).
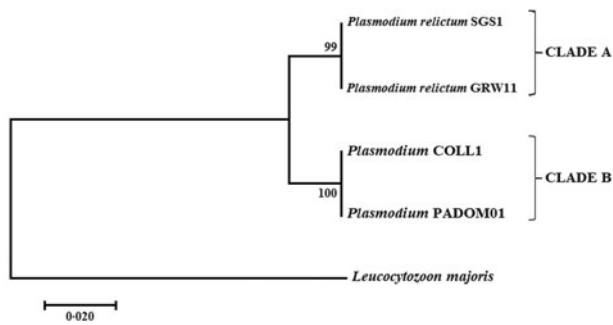
Fig. 1. Bootstrap consensus tree inferred from 10 000 replications for the *Plasmodium* lineages found in house sparrows.

Fig. 2. Percentage of *Cx. pipiens* head-thoraxes and saliva with presence of *Plasmodium* DNA from parasites of clades A (red) and B (blue). Statistically significant differences are indicated with an asterisk (*). NS means non-significant differences.

Fig. 3. Proportion of *Cx. pipiens* that survived until 13 dpe to *Plasmodium* parasites of clade A (red line), clade B (blue line) or control (black line). The shaded areas comprise the standard errors.

Parasite development in mosquitoes

Overall, 28 and 17 *Plasmodium*-infected house sparrows were exposed to 1713 *Cx. pipiens* and 974 *Ae. caspius*, respectively. Of these, 183 (10.7%) *Cx. pipiens* and 112 (11.5%) *Ae. caspius* fed on bird blood (Table 1). One house sparrow infected by the *Plasmodium* lineage PADOM01 was not bitten by any *Cx. pipiens*. *Plasmodium* infection status in the head-thorax was analysed for 126 *Cx. pipiens* fed on 27 infected birds (19 infected by parasites of clade A and 8 infected by parasites of clade B). Fifty-one (40.5%; $N = 126$) *Cx. pipiens* were positive for *Plasmodium* in the head-thorax. Eleven out of these 51 mosquitoes (21.6%) had *Plasmodium* DNA in their saliva. For *Ae. caspius*, *Plasmodium* infection status was analysed in the head-thorax of 45 mosquitoes fed on 12 infected birds (10 infected by parasites of clade A and 2 infected by parasites of clade B). None of the *Ae. caspius* that fed on three birds infected by *Plasmodium* SGS1, one bird infected by *Plasmodium* COLL1 and one bird infected by *Plasmodium* PADOM01 survived until 13 dpe. None of the 45 head-thoraxes of *Ae. caspius* analysed showed evidence of *Plasmodium* infection (Table 1).

Parasites were detected in the head-thorax of 37.6% ($N = 93$) and 48.8% ($N = 33$) *Cx. pipiens* fed on birds infected by *Plasmodium* lineages of clades A and B, respectively. Bird parasite load positively affected the prevalence of *Plasmodium* in the head-thorax of *Cx. pipiens* [estimate (est) = 0.86, $Z = 2.99$, $P = 0.003$], while parasite prevalence did not differ between clades (est = 0.42, $Z = 0.98$, $P = 0.33$; Fig. 2). By contrast, a higher prevalence of *Plasmodium* clade B (21.2%) than clade A (4.3%) was found in *Cx. pipiens* saliva (est = 1.81, $Z = 2.68$, $P = 0.007$; Fig. 2), while non-significant associations were found with parasite load in birds' blood (est = 0.52, $Z = 1.40$, $P = 0.16$). All *Plasmodium* lineages infecting house sparrows were detected in mosquito saliva and the same *Plasmodium* lineages were found in the head-thorax and saliva of each mosquito.

Mosquito survival

We monitored the survival up to 13 dpe of 180 *Cx. pipiens* fed on infected birds and 80 *Cx. pipiens* fed on 10 uninfected control birds. Fifty-one mosquitoes died before 13 dpe. Of them, 33.3% fed on birds infected with *Plasmodium* parasites of clade A, while only 8.3% fed on birds infected with parasite lineages of clade B. From those mosquitoes fed on uninfected control birds, 32.5% died before 13 dpe. The survival of mosquitoes did not depend on the bird infection status ($Z = -1.42$, $P = 0.16$), the host infection intensity ($Z = 1.31$, $P = 0.19$) or the mosquito age ($Z = 0.9$; $P = 0.4$). However, when considering the identity of *Plasmodium* parasites instead of the bird infection status, *Cx. pipiens* fed on birds infected by parasites of clade B survived longer than those fed on birds infected by clade A ($Z = 2.23$;
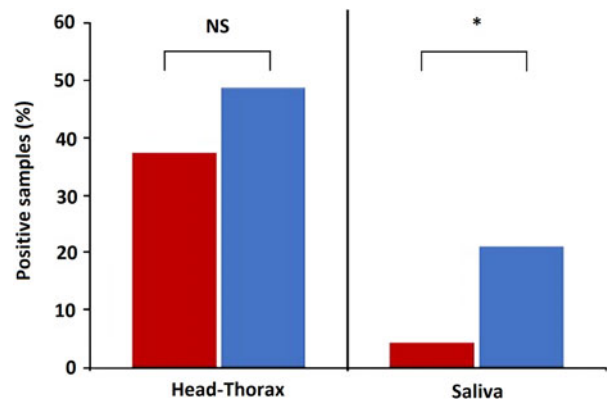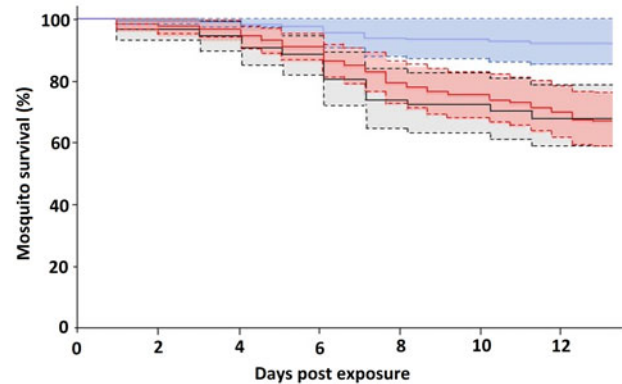
$P = 0.03$; Fig. 3), and on un-infected control birds ($Z = 2.83$; $P = 0.005$), while parasite load in the bird host ($Z = 1.39$, $P = 0.16$) and mosquito age ($Z = 0.69$; $P = 0.49$) did not affect mosquito survival.

Plasmodium transmission risk

Both parameters, i.e. transmission rate and vector survival, were affected by the parasite clade and consequently both clades differed in their risk of transmission. Mosquitoes fed on birds infected by *Plasmodium* lineages of clade B had a higher daily survival probability (P) than those fed on birds infected by *Plasmodium* lineages of clade A (daily survival probability = 0.99 and 0.97, respectively). Moreover, the transmission rate (c) of *Plasmodium* lineages of clade B by *Cx. pipiens* was higher than the transmission rate of *Plasmodium* lineages of clade A (transmission rate = 0.21 and 0.04, respectively). Consequently, *Plasmodium* parasites clade A showed a 20.3 times lower relative transmission risk number than those of clade B ($R_{0,rel} = 18.48$ and 0.9 for clades B and A, respectively).

Discussion

The successful transmission of mosquito-borne parasites largely depends on the availability of competent vector species in the area (Beerntsen *et al.*, 2000). Although mosquitoes of the genus *Culex* are considered the main vectors of avian *Plasmodium*, molecular detection of different *Plasmodium* lineages in other

mosquito genera, such as *Anopheles*, *Aedes* and *Lutzia* (Kimura et al., 2010; Santiago-Alarcón et al., 2012; Ferraguti et al., 2013; Schoener et al., 2017), suggests that avian *Plasmodium* spp. are not tightly coevolved with mosquito species. However, our results indicate that while *Cx. pipiens* being capable of transmitting the four *Plasmodium* lineages we isolated from birds, *Ae. caspius* does not, which suggests the existence of parasite/insect related mechanisms that prevents the successful development and subsequent transmission of the parasite by some mosquito species (Hardy et al., 1983; Black and Moore, 1996). The insect midgut represents a strong barrier, being able to dramatically reduce the number of viable parasites from those initially ingested (Abraham and Jacobs-Lorena, 2004; Siden-Kiamos et al., 2006). In addition, *Plasmodium* penetrates the midgut intracellular epithelium by a complex mechanism involving numerous proteins of the membrane (Povelones et al., 2009). Thus, potential differences in the presence of these proteins between mosquito species could explain the inability of avian *Plasmodium* to develop in *Ae. caspius* mosquitoes. Furthermore, differences in the immune response against parasites or midgut microbiota between mosquito species may affect parasite development (Azambuja et al., 2005; Weiss and Aksoy, 2011). Our results strongly support that detection of *Plasmodium* DNA in a particular mosquito species body is not a good indicator of the species' vector competence, since *Plasmodium* DNA was previously detected, even at high prevalence, in *Ae. caspius* (Ferraguti et al., 2013; Schoener et al., 2017). Although mosquito head-thorax and saliva were sampled at 13 dpe, a period that exceeds the time needed for different *Plasmodium* spp. to develop in the mosquito salivary glands (Valkiūnas, 2005; LaPointe et al., 2010), there may exist mosquito–parasite combinations that require different pre-patent periods for parasite development. This could also explain the absence of *Plasmodium* DNA in *Ae. caspius*.

Despite *Cx. pipiens* is a competent vector of avian *Plasmodium*, we found that its competence for the parasite transmission differed between the two clades. We found a reduced impact on mosquito survival and a higher parasite prevalence in mosquito saliva of clade B lineages (COLL1 and PADOM01), while for clade A (SGS1 and GRW11), we found a higher impact on mosquito survival and lower prevalence in mosquito saliva. As a result, the vector competence of *Cx. pipiens* for clade B parasites was much higher than for those of clade A. However, these differences could be the result of unequal parasite development in the mosquitoes. It is possible that both clades differ in the time required to develop and reach the salivary glands, as has been reported between *Plasmodium* parasite species (LaPointe et al., 2010; Palinauskas et al., 2016), with parasites of clade B producing sporozoites faster than those of clade A. This could be due to different pre-patent periods of parasite development in the mosquito. In fact, Maier (1973) found sporozoites of *P. cathemerium* in the salivary glands of *Cx. pipiens* mosquitoes at 7 dpe, and *P. relictum* sporozoites in the salivary glands of mosquitoes have been recorded as early as 4 and 5 dpe (Rosen and Reeves, 1954; Work et al., 1990). However, Kazlauskienė et al. (2013) did not find sporozoites of the *P. relictum* lineages SGS1 and GRW11 (clade A in this study) until 14 dpe in the salivary glands of *Cx. pipiens* mosquitoes (presence of sporozoites was analysed at 12 dpe, but not at 13 dpe). In addition to parasite identity, the parasite load of the vertebrate host may largely determine the success of parasite development in the insect vector and, potentially, its ability for being transmitted. In humans, *Plasmodium* gametocytaemia was positively associated with the mosquito infection rates (Bousema and Drakeley et al., 2011). In avian *Plasmodium*, however, non-significant associations between host parasitaemia and oocyst prevalence have been found (Pigeault and Villa, 2018). However, the interaction of

bird hosts and mosquitoes could potentially affect the within-host dynamics of *Plasmodium* and therefore, its transmission to mosquitoes. For example, it has been shown that daily variations in mosquito activity are correlated with bird parasitaemia and that mosquito bites increase *P. relictum* replication in the birds both in the acute and in the chronic phases of the infection (Pigeault et al., 2018). Our results suggest that although *Plasmodium* load in the avian host facilitates the detection of parasite DNA in the mosquito head-thorax, the successful infection and transmission of *Plasmodium* into mosquito saliva was not directly related to infection load in the bird host. Consequently, the final development of parasites in mosquito saliva may be modulated by other factors, including specific mosquito–parasite assemblages.

The costs of *Plasmodium* infection for mosquito survival remain a subject of intense debate (Ferguson and Read, 2002; Martínez-de la Puente et al., 2018). Vézilier et al. (2012) reported a decreased fecundity and an increased longevity of mosquitoes fed on infected birds, while Pigeault and Villa (2018) did not find any association between bird parasite load and mosquito survival. However, these studies focused on the interaction between *Cx. pipiens* and *P. relictum*. In our study, we found that mosquitoes fed on birds infected by *Plasmodium* clade B had higher survival than those fed on birds infected by clade A (*P. relictum*) or un-infected birds. Differences in the level of virulence between avian *Plasmodium* lineages on mosquitoes are currently unknown. Nonetheless, a differential impact on bird hosts of parasite lineages/morphospecies has been reported. For instance, Lachish et al. (2011) found that *P. relictum* had a lower virulence on birds than *P. circumflexum*. Our results suggest that this may also occur in mosquitoes, with differential cost [i.e. energetic cost (Hurd et al., 2005); survival, this study] imposed by different species/lineages of *Plasmodium*. Although birds infected by both clades (clades A and B) showed similar *Plasmodium* intensities of infection, mosquitos feeding on birds with high intensities of infection die sooner (Gutiérrez-López et al., 2019a), probably due to the cost of *Plasmodium* infection on mosquito survival.

We found that *Plasmodium* transmission risk differed between parasite clades mainly due to their differential impact on mosquito survival and transmission rate (i.e. presence in saliva). Lineages of *P. relictum* (clade A) had a higher virulence on mosquitoes and also showed a lower transmission rate than parasites of clade B. Consequently, transmission of *Plasmodium* was less effective when mosquitoes fed on birds infected by lineages of clade A than when infected by clade B. In addition to the variables measured here, the epidemiology of vector-borne parasites depends on a number of factors, such as host density (Gubbins et al., 2008), host recovery rate (Macdonald, 1955) and vector density (Hartemink et al., 2011). In addition, our estimation of the parasite transmission risk should be considered with caution as we monitored the survival rate of mosquitoes until 13 dpe, while epidemiological models should consider the whole lifespan of individuals. In spite of these limitations, our results provide a first step towards the identification of the consequences of parasite identity for avian malaria epidemiology, a topic that has been traditionally neglected. Interestingly, *P. relictum* (clade A) is considered a generalist parasite infecting more than 300 species of birds belonging to 11 different orders worldwide, being transmitted by 20 different species of mosquitoes (Valkiūnas et al., 2018). However, recent studies have found that generalist parasites may also show a specialized behaviour in certain, phylogenetically related host species within their host range (Svensson-Coelho et al., 2016; Huang et al., 2018). These findings suggest that the generalist/specialist character of a parasite should not be solely based on its host range, which may in turn vary among different host and parasite communities (Svensson-Coelho et al., 2016), and highlights that phylogenetic relationships among hosts

must be considered when determining the specificity of a particular parasite (Huang *et al.*, 2018). However, all such studies refer to the generalist/specialist continuum from a vertebrate host perspective, while little information is available on virulence and specialization on the vectors. Our results showed a higher efficacy of transmission by *Cx. pipiens* of parasites of clade B as compared with those of clade A. This could be due to the generalist character of *P. relictum*, which may decrease its fitness when transmitted to *Cx. pipiens*, but not when infecting birds (Hellgren *et al.*, 2009). Further studies are necessary to understand the range of vectors successfully transmitting the different species of avian *Plasmodium*.

In conclusion, results from this study confirm the existence of inter- and intra-specific differences in the ability of *Plasmodium* lineages to develop in different mosquito species. While some mosquitoes such as *Ae. caspius* were refractory to parasite development, *Cx. pipiens* play a key role in the transmission of avian *Plasmodium* by regulating, for instance, its temporal (e.g. Lalubin *et al.*, 2014) and spatial dynamics (Martínez-de la Puente *et al.*, 2016) in both natural and anthropized environments. Here, we add valuable information on the competence of *Cx. pipiens* for the transmission of four *Plasmodium* lineages with active circulation in Europe. Nevertheless, the identity of each vector–parasite assemblage may modulate the transmission success of *Plasmodium* lineages through differences in the parasite transmission rate in the mosquito and the costs of infection on mosquito survival. Consequently, *Cx. pipiens* was better at transmitting lineages related to *P. cathemerium* than *P. relictum* due to a lower impact on mosquito survival and higher ability to replicate and reach mosquito saliva of the former. Understanding how *Plasmodium* virulence in the host and the vector interact may be crucial to understand the maintenance and abundance of the different *Plasmodium* species and lineages in the wild.

References

Abraham EG and Jacobs-Lorena M (2004) Mosquito midgut barriers to malaria parasite development. *Insect Biochemistry and Molecular Biology* **34**, 667–671.

Azambuja P, Garcia ES and Ratcliffe NA (2005) Gut microbiota and parasite transmission by insect vectors. *Trends in Parasitology* **21**, 568–572.

Balenghien T, Fouque F, Sabatier P and Bicout DJ (2006) Horse-, bird-, and human-seeking behavior and seasonal abundance of mosquitoes in a West Nile virus focus of southern France. *Journal of Medical Entomology* **43**, 936–946.

Bates D, Maechler M, Bolker B and Walker S (2015) Fitting linear mixed-effects models using lme4. *Journal of Statistical Software* **67**, 1–48.

Beerntsen BT, James AA and Christensen BM (2000) Genetics of mosquito vector competence. *Microbiology and Molecular Biology Reviews* **64**, 115–137.

Bensch S, Hellgren O and Pérez-Tris J (2009) Malavi: a public database of malaria parasites and related haemosporidians in avian hosts based on mitochondrial cytochrome b lineages. *Molecular Ecology Resources* **9**, 1353–1358.

Black WCIV and Moore CG (1996) Population biology as a tool for studying vector-borne diseases. In Beaty BJ and Marquardt WC (eds), *The Biology of Disease Vectors*. Colorado, USA: University Press of Colorado, pp. 393–416.

Bousema T and Drakeley C (2011) Epidemiology and infectivity of Plasmodium falciparum and Plasmodium vivax gametocytes in relation to malaria control and elimination. *Clinical microbiology reviews* **24**, 377–410.

Cornet S, Nicot A, Rivero A and Gandon S (2013) Both infected and uninfected mosquitoes are attracted toward malaria infected birds. *Malaria Journal* **12**, 179.

Cornet S, Nicot A, Rivero A and Gandon S (2014) Evolution of plastic transmission strategies in avian malaria. *PLoS Pathogens* **10**, e1004308.

Delhaye J, Aletti C, Glaizot O and Christe P (2016) Exposure of the mosquito vector Culex pipiens to the malaria parasite Plasmodium relictum: effect of infected blood intake on immune and antioxidant defences, fecundity and survival. *Parasites and Vectors* **9**, 616.

Dubrulle M, Mousson L, Moutailler S, Vazeille M and Failloux AB (2009) Chikungunya virus and *Aedes* mosquitoes: saliva is infectious as soon as two days after oral infection. *PLoS ONE* **4**, e5895.

Fallon SM, Bermingham E and Ricklefs RE (2005) Host specialization and geographic localization of avian malaria parasites: a regional analysis in the *Lesser antilles*. *American Naturalist* **165**, 466–480.

Ferguson HM and Read AF (2002) Why is the effect of malaria parasites on mosquito survival still unresolved? *Trends in Parasitology* **18**, 256–261.

Ferraguti M, Martínez-de la Puente J, Muñoz J, Roiz D, Ruiz S, Soriguer RC and Figuerola J (2013) Avian *Plasmodium* in *Culex* and *Ochlerotatus* mosquitoes from southern Spain: effects of season and host-feeding source on parasite dynamics. *PLoS ONE* **8**, e66237.

Ferraguti M, Martínez-de la Puente J, Bensch S, Roiz D, Ruiz S, Viana DS, Soriguer RC and Figuerola J (2018) Ecological determinants of avian malaria infections: an integrative analysis at landscape, mosquito and vertebrate community levels. *Journal of Animal Ecology* **87**, 727–740.

Franks SJ, Pratt PD and Tsutsui ND (2011) The genetic consequences of a demographic bottleneck in an introduced biological control insect. *Conservation Genetics* **12**, 201–211.

Gering E and Atkinson CT (2004) A rapid method for counting nucleated erythrocytes on stained blood smears by digital image analysis. *Journal of Parasitology* **90**, 879–881.

Goddard LB, Roth AE, Reisen WK and Scott TW (2002) Vector competence of California mosquitoes for West Nile virus. *Emerging Infectious Diseases* **8**, 1385–1391.

Golenda CF, Burge R and Schneider I (1992) Plasmodium falciparum and P. berghei: detection of sporozoites and the circumsporozoite proteins in the saliva of Anopheles stephensi mosquitoes. *Parasitology Research* **78**, 563–569.

Gubbins S, Carpenter S, Baylis M, Wood JL and Mellor PS (2008) Assessing the risk of bluetongue to UK livestock: uncertainty and sensitivity analyses of a temperature-dependent model for the basic reproduction number. *Journal of the Royal Society Interface* **5**, 363–371.

Gutiérrez-López R, Martínez-de la Puente J, Gangoso L, Soriguer RC and Figuerola J (2015) Comparison of manual and semi-automatic DNA extraction protocols for the barcoding characterization of hematophagous louse flies (Diptera: Hippoboscidae). *Journal of Vector Ecology* **40**, 11–15.

Gutiérrez-López R, Martínez-de la Puente J, Gangoso L, Yan J, Soriguer RC and Figuerola J (2016) Do mosquitoes transmit the avian malaria-like parasite Haemoproteus? An experimental test of vector competence using mosquito saliva. *Parasites and Vectors* **9**, 609.

Gutiérrez-López R, Martínez-de la Puente J, Gangoso L, Yan J, Soriguer RC and Figuerola J (2019a) Infection load influences *Plasmodium* transmission risk due to their effects on mosquito survival. *Scientific Reports* **9**, 8782.

Gutiérrez-López R, Martínez-de la Puente J, Gangoso L, Soriguer RC and Figuerola J (2019b) Effects of host sex, body mass and infection by avian *Plasmodium* on the biting rate of two mosquito species with different feeding preferences. *Parasites and Vectors* **12**, 87.

Gutiérrez-López R, Bialosuknia SM, Ciota AT, Montalvo T, Martínez-de la Puente J, Gangoso L, Figuerola J and Kramer LD (2019c) Vector competence of *Aedes caspius* and *Ae. albopictus* mosquitoes for Zika virus, Spain. *Emerging Infectious Diseases* **25**, 346–348.

Hardy JL, Houk EJ, Kramer LD and Reeves WC (1983) Intrinsic factors affecting vector competence of mosquitoes for arboviruses. *Annual Review of Entomology* **28**, 229–262.

Hartemink N, Vanwambeke SO, Heesterbeek H, Rogers D, Morley D, Pesson B, Davies C, Mahamdallie S and Ready P (2011) Integrated mapping of establishment risk for emerging vector-borne infections: a case study of canine leishmaniasis in southwest France. *PLoS ONE* **6**, e20817.

Hellgren O, Waldenström J and Bensch S (2004) A new PCR assay for simultaneous studies of *Leucocytozoon*, *Plasmodium*, and *Haemoproteus* from avian blood. *Journal of Parasitology* **90**, 797–802.

Hellgren O, Pérez-Tris J and Bensch S (2009) A jack-of-all-trades and still a master of some: prevalence and host range in avian malaria and related blood parasites. *Ecology* **90**, 2840–2849.

Huang X, Ellis VA, Jönsson J and Bensch S (2018) Generalist haemosporidian parasites are better adapted to a subset of host species in a multiple host community. *Molecular Ecology* **27**, 4336–4346.

Hurd H, Taylor PJ, Adams D, Underhill A and Eggleston P (2005) Evaluating the costs of mosquito resistance to malaria parasites. *Evolution* **59**, 2560–2572.

Ishtiaq F, Guillaumot L, Clegg SM, Phillimore AB, Black RA, Owens IPF, Mundy NI and Sheldon BC (2008) Avian haematozoan parasites and their associations with mosquitoes across Southwest Pacific Islands. *Molecular Ecology* **17**, 4545–4555.

Kazlauskienė R, Bernotienė R, Palinauskas V, Iezhova TA and Valkiūnas G (2013) *Plasmodium relictum* (lineages pSGS1 and pGRW11): complete synchronous sporogony in mosquitoes *Culex pipiens pipiens*. *Experimental Parasitology* **133**, 454–461.

Kimura M, Darbro JM and Harrington LC (2010) Avian malaria parasites share congeneric mosquito vectors. *Journal of Parasitology* **96**, 144–151.

Kumar S, Stecher G and Tamura K (2016) MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution* **33**, 1870–1874.

Lachish S, Knowles SC, Alves R, Wood MJ and Sheldon BC (2011) Infection dynamics of endemic malaria in a wild bird population: parasite species-dependent drivers of spatial and temporal variation in transmission rates. *Journal of Animal Ecology* **80**, 1207–1216.

Lagisz M, Port G and Wolff K (2011) Living in a jar: genetic variation and differentiation among laboratory strains of the red flour beetle. *Journal of Applied Entomology* **135**, 682–692.

Lalubin F, Delédevant A, Glaizot O and Christe P (2013) Temporal changes in mosquito abundance (Culex pipiens), avian malaria prevalence and lineage composition. *Parasites & vectors* **6**, 307.

Lalubin F, Deledevant A, Glaizot O and Christe P (2014) Natural malaria infection reduces starvation resistance of nutritionally stressed mosquitoes. *Journal of Animal Ecology* **83**, 850–857.

LaPointe DA, Goff ML and Atkinson CT (2010) Thermal constraints to the sporogonic development and altitudinal distribution of avian malaria *Plasmodium relictum* in Hawai'i. *Journal of Parasitology* **96**, 318–324.

Lefévre T, Vantaux A, Dabire KR, Mouline K and Cohuet A (2013) Non-genetic determinants of mosquito competence for malaria parasites. *PLoS Pathogens* **9**, e1003365.

Lover AA and Coker RJ (2015) Do mixed infections matter? Assessing virulence of mixed-clone infections in experimental human and murine malaria. *Infection, Genetics and Evolution* **36**, 82–91.

Macdonald G (1955) The measurement of malaria transmission. *Proceedings of the Royal Society of Medicine* **48**, 295–301.

Maier WA (1973) Über die mortalität von *Culex pipiens Fatigans* nach infektion mit *Plasmodium cathemerium*. *Zeitschrift fur Parasitenkunde* **41**, 11–28.

Martínez-de la Puente J, Ferraguti M, Ruiz S, Roiz D, Soriguer RC and Figuerola J (2016) *Culex pipiens* forms and urbanization: effects on blood feeding sources and transmission of avian Plasmodium. *Malaria Journal* **15**, 589.

Martínez-de la Puente J, Gutiérrez-López R and Figuerola J (2018) Do avian malaria parasites reduce vector longevity? *Current Opinion in Insect Science* **28**, 113–117.

Muñoz J, Ruiz S, Soriguer RC, Alcaide M, Viana DS, Roiz D, Vázquez A and Figuerola J (2012) Feeding patterns of potential West Nile virus vectors in south-west Spain. *PLoS ONE* **7**, e39549.

Paaijmans KP, Imbahale SS, Thomas MB and Takken W (2010) Relevant microclimate for determining the development rate of malaria mosquitoes and possible implications of climate change. *Malaria Journal* **9**, 196.

Palinauskas V, Žiegytė R, Iezhova TA, Ilgūnas M, Bernotienė R and Valkiūnas G (2016) Description, molecular characterisation, diagnostics and life cycle of *Plasmodium elongatum* (lineage pERIRUB01), the virulent avian malaria parasite. *International Journal for Parasitology* **46**, 697–707.

Pérez-Tris J, Hellgren O, Križanauskienė A, Waldenström J, Secondi J, Bonneaud C, Fjeldså J, Hasselquist D and Bensch S (2007) Within-host speciation of malaria parasites. *PLoS ONE* **2**, e235.

Pigeault R and Villa M (2018) Long-term pathogenic response to *Plasmodium relictum* infection in *Culex pipiens* mosquito. *PLoS ONE* **13**, e0192315.

Pigeault R, Vézilier J, Cornet S, Zélé F, Nicot A, Perret P, Gandon S and Rivero A (2015) Avian malaria: a new lease of life for an old experimental model to study the evolutionary ecology of *Plasmodium*. *Philosophical Transactions of the Royal Society B: Biological Sciences* **370**, 20140300.

Pigeault R, Caudron Q, Nicot A, Rivero A and Gandon S (2018) Timing malaria transmission with mosquito fluctuations. *Evolution Letters* **2**, 378–389.

Povelones M, Waterhouse RM, Kafatos FC and Christophides GK (2009) Leucine-rich repeat protein complex activates mosquito complement in defense against *Plasmodium* parasites. *Science* **324**, 258–261.

R Core Team (2016) *R: A Language and Environment for Statistical Computing*. Vienna, Austria: R Foundation for Statistical Computing.

Rosen L and Reeves WC (1954) Studies on avian malaria in vectors and hosts of encephalitis in Kern County, California. *American Journal of Tropical Medicine and Hygiene* **3**, 704–708.

Ross R (1911) *The Prevention of Malaria*. London, UK: John Murray.

Sachs J and Malaney P (2002) The economic and social burden of malaria. *Nature* **415**, 680.

Santiago-Alarcon D, Palinauskas V and Schaefer HM (2012) Diptera vectors of avian Haemosporidian parasites: untangling parasite life cycles and their taxonomy. *Biological Reviews* **87**, 928–964.

Schaffner E, Angel G, Geoffroy B, Hervy JP, Rhaiem A and Brunhes J (2001) *The Mosquitoes of Europe: An Identification and Training Programme*. Montpellier, France: IRD Editions.

Schoener E, Uebleis SS, Butter J, Nawratil M, Cuk C, Flechl E, Kothmayer M, Obwaller AG, Zechmeister T, Rubel F, Lebl K, Zittra C and Fuehrer HP (2017) Avian *Plasmodium* in Eastern Austrian mosquitoes. *Malaria Journal* **16**, 389.

Siden-Kiamos I, Ecker A, Nybäck S, Louis C, Sinden RE and Billker O (2006) *Plasmodium berghei* calcium-dependent protein kinase 3 is required for ookinete gliding motility and mosquito midgut invasion. *Molecular Microbiology* **60**, 1355–1363.

Svensson-Coelho M, Loiselle BA, Blake JG and Ricklefs RE (2016) Resource predictability and specialization in avian malaria parasites. *Molecular Ecology* **25**, 4377–4391.

Therneau TM and Lumley T (2014) Survival: survival analysis, including penalised likelihood. Available at http://cran.r-project.org/package=survival.

Valkiūnas G (2005) *Avian Malaria Parasites and Other Haemosporidia*. Florida, USA: CRC Press.

Valkiūnas G (2011) Haemosporidian vector research: marriage of molecular and microscopical approaches is essential. *Molecular Ecology* **20**, 3084–3086.

Valkiūnas G, Ilgūnas M, Bukauskaitė D, Fragner K, Weissenböck H, Atkinson CT and Iezhova TA (2018) Characterization of *Plasmodium relictum*, a cosmopolitan agent of avian malaria. *Malaria Journal* **17**, 184.

Vézilier J, Nicot A, Gandon S and Rivero A (2012) *Plasmodium* infection decreases fecundity and increases survival of mosquitoes. *Proceedings of the Royal Society B: Biological Sciences* **279**, 4033–4041.

Weiss B and Aksoy S (2011) Microbiome influences on insect host vector competence. *Trends in Parasitology* **27**, 514–522.

Work TM, Washino RK and Van Riper III C (1990) Comparative susceptibility of *Culex tarsalis*, *Anopheles franciscanus* and *Culiseta inornata* (Diptera: Culicidae) to *Plasmodium relictum* (Haemosporidia: Plasmodiiae). *Journal of Medical Entomology* **27**, 68–71.

Yan J, Martínez-de la Puente J, Gangoso L, Gutiérrez-López R, Soriguer R and Figuerola J (2018) Avian malaria infection intensity influences mosquito feeding patterns. *International Journal for Parasitology* **48**, 257–264.