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PhD Thesis

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Impact of the interactions between hosts, vectors and pathogens on the transmission of avian malaria and flavivirus by mosquitoes

Memoria presentada por el Licenciado en Biología, Rafael Gutiérrez López, para optar al título de Doctor por la Universidad de Sevilla

Fdo. Rafael Gutiérrez López

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CERTIFICAN:

Que los trabajos de investigación desarrollados en la Memoria de Tesis Doctoral "*Impact of the interactions between hosts, vectors and pathogens on the transmission of avian malaria and flavivirus by mosquitoes*", son aptos para ser presentados por el Licenciado Rafael Gutiérrez López ante el Tribunal que en su día se designe, para aspirar al grado de Doctor por la Universidad de Sevilla.

Y para que así conste, y en cumplimiento de las disposiciones legales vigentes, firman el presente documento en Sevilla, a 15 de mayo de 2018.

Directores:

Fdo. Jordi Figuerola Borras

Fdo. Josué Martínez de la Puente

Fdo. Laura Gangoso de la Colina

Fdo. Javier Balbontín Arenas (Tutor)

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Mosquito and bird sampling were conducted with all the necessary permits from the regional Departments of the Environments (Consejería de Medio Ambiente, Junta de Andalucía). Entomological and birds sampling on private land were conducted with all the necessary permits and consent, and in the presence of owners.

“No hay nada más maravilloso que ser un científico,
en ninguna parte preferiría estar más que en mi laboratorio,
manchando mi ropa y cobrando por jugar”

-Marie Curie-

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ABSTRACT

Vector-borne pathogens cause important diseases such as malaria and are nowadays a major public health concern, because they cause human –and animal- fatalities worldwide and have a significant impact on local economies. Factors associated to global change, such as habitat alteration and introduction of invasive species, have largely contributed to the spread of potential insect vectors and the pathogens they are able to transmit, thus creating novel epidemiological scenarios. Therefore, it becomes essential to study the factors that modulate the transmission risk of these disease agents involving the interactions between vertebrate hosts (humans and other animals), pathogens and insect vectors in natural ecosystems.

In this thesis, I used a multidisciplinary approach combining molecular tools, experimental bioassays and statistical analyses to assess the ecological and evolutionary factors that affect the transmission success of two mosquito-borne pathogens. In particular, I considered the interactions between insect vectors, vertebrate hosts, the avian malaria parasites and the flavivirus Zika virus. I focused on two major steps directly influencing the pathogen transmission success: i) the contact rate between mosquitoes and infected/susceptible vertebrate hosts and ii) the development of the pathogen in the mosquito and its consequences on the pathogen transmission risk.

To do that, first I tested the potential causes underlying differences in the biting patterns of mosquito species. I exposed two bird species to two mosquito species to determine the role of mosquito species identity and effect of three host-related factors on host-vector contact rates (i.e. body mass, gender, and infection status by avian malaria). I found clear interspecific differences in the biting rates of mosquitoes, which were also influenced by variation in hosts' traits, although these effects differed depending on the particular mosquito-host assemblage. Therefore, the biting patterns of mosquitoes are far from being generalizable.

Secondly, I assessed the vector competence of different mosquito species for the transmission of Zika virus and avian malaria using mosquito saliva. I found that the ability of mosquito-borne pathogens to develop in mosquitoes differed between insect species, which may be the result of complex co-evolutionary processes. In addition, I assessed the consequences of parasite development in the mosquito vectors and their implications for

the pathogen transmission risk. I found that host parasite load and parasite identity play affect the impact of parasites on mosquito longevity finally determining the transmission risk of the parasites.

With this information mostly derived from studies under controlled conditions, I assessed the importance of environmental conditions affecting the host-parasite-vector assemblages in the wild. I found that habitat characteristics, which determine the existence and abundance of insect vectors, and host related factors (i.e. immune-competence) determines the prevalence of avian malaria parasites in insular ecosystems.

Altogether, in this thesis I identified key factors affecting the transmission success of vector-borne pathogen affecting humans or wildlife allowing a better understanding the complex transmission dynamics of vector-borne pathogens.

RESUMEN

Los patógenos transmitidos por vectores que causan enfermedades como la malaria son a día de hoy un importante problema de salud pública, ya que causan numerosas muertes humanas y animales a nivel mundial teniendo un gran impacto en la economía local.

Factores asociados al cambio global, así como la alteración del hábitat y la introducción de especies invasoras, han contribuido en gran medida a la expansión de posibles insectos vectores y de los patógenos que pueden transmitir, creándose nuevos escenarios epidemiológicos. Por lo tanto, es esencial estudiar aquellos factores que influyen en el riesgo de transmisión de estos patógenos considerando las interacciones entre los huéspedes vertebrados (humanos y otros animales), los patógenos insectos vectores en los ecosistemas naturales.

En esta tesis, mediante un enfoque multidisciplinario que combina herramientas moleculares, bioensayos experimentales y análisis estadísticos, se evalúan los factores ecológicos y evolutivos que afectan al éxito de transmisión de dos patógenos transmitidos por mosquitos. En particular, se han consideraron las interacciones entre los parásitos de la malaria aviar y el virus del Zika con sus insectos vectores y hospedadores vertebrados. En concreto, me centro en dos procesos principales que influyen directamente en el éxito de la transmisión de patógenos: i) la tasa de contacto entre mosquitos y hospedadores vertebrados infectados/ susceptibles de serlo y ii) el desarrollo del patógeno en el mosquito y sus consecuencias sobre el riesgo de transmisión de éstos.

Para ello, primero he estudiado las posibles causas que subyacen a las diferencias en los patrones de alimentación de las especies de mosquitos. Se expusieron dos especies de pájaros a dos especies de mosquitos para determinar el papel que tenía la especie de mosquito y el efecto de tres factores relacionados con el hospedador vertebrado sobre las tasas de contacto entre el hospedador y el vector (masa corporal, género e infección por parásitos de la malaria aviar). Encontré claras diferencias inter-específicas en las tasas de picadura de los mosquitos, que también se vieron influenciadas por la variación en los rasgos de los hospedadores, aunque estos efectos difirieron dependiendo de la asociación mosquito-hospedador. Por lo tanto, los patrones de alimentación de los mosquitos parecen estar lejos de ser generalizables.

En segundo lugar, he estudiado la competencia vectorial de diferentes especies de mosquitos para la transmisión del virus del Zika y de parásitos de la malaria aviar utilizando la saliva del mosquito. Descubrí que la capacidad de desarrollo de los patógenos difería entre las especies de mosquitos, pudiendo ser el resultado de complejos procesos co-evolutivos. Además, he evaluado las consecuencias que tiene el desarrollo de los parásitos en los mosquitos en relación al riesgo de transmisión de éstos. También encontré que tanto la carga parasitaria del hospedador vertebrado como la identidad del parásito afectan a la longevidad de los mosquitos y consecuentemente determina el riesgo de transmisión de los parásitos.

Con esta información, derivada principalmente de estudios en laboratorio, bajo condiciones controladas, he evaluado cómo afectan las condiciones ambientales a las asociaciones entre hospedador-parásito-vector en la naturaleza. Descubrí que las características del hábitat, las cuales van a determinan la existencia y abundancia de insectos vectores, así como los factores relacionados con el huésped (inmunocompetencia), determinan la prevalencia de parásitos de la malaria aviar en los ecosistemas insulares.

En general, en esta tesis, he identificado los factores claves que afectan al éxito de transmisión de los patógenos transmitidos por insectos vectores que afectan a la salud humana o animal, permitiendo un mejor entendimiento de la complejidad de la dinámica de transmisión de estos patógenos.

GENERAL SECTION

Introduction

Factors associated to global change, including climate change, habitat alteration and the introduction of invasive species have largely contributed to the emergence and re-emergence of different diseases of public health relevance (Jones *et al.* 2008; Morens & Fauci, 2013). In addition, the increase of international travels and trades also favour the spread of disease agents across the globe (Patz *et al.* 2000; Kilpatrick & Randolph, 2012). Some vector-borne diseases such as malaria, filariasis, Dengue fever, West Nile fever or Chikungunya fever are nowadays a major health concern, since they cause human fatalities worldwide and have a significant economic impact on local economies (Goddard, 2008). Many of these infectious diseases have a zoonotic origin, being transmitted by insect vectors, such as mosquitoes, ticks, or fleas, from animals (wildlife, livestock) to humans (Vorou *et al.* 2007). One example of that is the re-emergent West Nile virus (WNV) that circulates naturally between birds, but occasionally affects humans and horses. Since its introduction in 1999 in North America, over 3 million people have been infected with WNV in the USA (Petersen *et al.* 2013). Moreover, the spread of potential insect vectors and/or the pathogens they can transmit create novel epidemiological scenarios, such as the transmission of locally circulating *Dirofilaria* parasites by invasive mosquito *Aedes albopictus* in Italy (Cancrini *et al.* 2003), or imported pathogens such as Dengue and Chikunguya viruses by *Ae. albopictus* in France (Gould *et al.* 2010; Marchand *et al.* 2013). Therefore, it becomes essential to study the interactions between vertebrate hosts (humans and other animals), insect vectors, and pathogens potentially transmitted in natural ecosystems to fully understand the ecology and evolution of the transmission of diseases.

Hosts and pathogens are involved in a permanent evolutionary arms race to maximize their own fitness (Thompson, 1998), with pathogens trying to increase their transmission success and hosts minimizing the costs of infections (de Roode *et al.* 2008). Nonetheless, due to their relative shorter life cycles, pathogens are usually one step forward with respect to their hosts. Under this co-evolutionary scenario, pathogens are usually locally and temporally adapted to their most common hosts. However, pathogens often come into contact with other uncommon hosts, potentially increasing their host-range and

occasionally producing outbreaks (Woolhouse *et al.* 2005). This arms race between pathogens and hosts becomes even more complicated for the case of vector-borne pathogens. Pathogen-vector interactions include different physiological, immunological, and genetic mechanisms allowing/hampering the development of the infection in the insect vector (Beerntsen *et al.* 2000). Thus, those genetic and phenotypic changes in pathogens allowing the adaptation to new hosts (i.e. vertebrate hosts and insect vectors) could affect their interactions with their common hosts (Escalante *et al.* 1998; Waldenstrom *et al*.. 2002; Ricklefs *et al.* 2002, 2004). For instance, a single point mutation associated with the ability of Chikungunya virus to develop in *Aedes albopictus* has been also associated with a reduction in the transmission efficiency by its natural vector *Aedes aegypti* (Lamballerie *et al.* 2008). Moreover, environmental conditions may influence these interactions finally determining the transmission dynamics of vector-borne pathogens (Ferraguti *et al.* 2018). In this thesis, I study the main factors potentially affecting the transmission dynamics of pathogens with ecological and public health importance (i.e. virus and protozoa) by assessing the role played by different potential vector species. In particular, I focus on two factors directly influencing the pathogen transmission success 1) the contact rate between insect vectors and infected/susceptible vertebrate hosts and 2) the development of the pathogen in the insect vector.

The basic reproductive number (R_0) is an epidemiological parameter frequently used to determine the transmission risk of pathogens (Smith *et al.* 2012). R_0 corresponds to the average number of secondary infections caused by an infectious host entering a naïve population. R_0 is estimated from models that incorporate basic information on the transmission process that includes, in the case of vector-borne pathogens, host selection by vectors, vector survival and the time necessary for the development of the pathogen in the insect vectors (Ross, 1911; Macdonald, 1955; Fig. 1). This is especially important for the case of multi-vector pathogens for which the relative importance of each vector species for pathogen transmission may differ (i.e. mosquitoes, see Chen *et al.* 1993; Goddard *et al.* 2002; Ciota *et al.* 2017). However, for many pathogen-vector assemblages this information is not available. In addition, most parameters available in the literature are derived from analyses of the interactions between insect colonies and pathogens maintained by serial

passes in the laboratory, which are known to strongly affect the virulence of vector-borne pathogens (Mackinnon & Read, 2004).

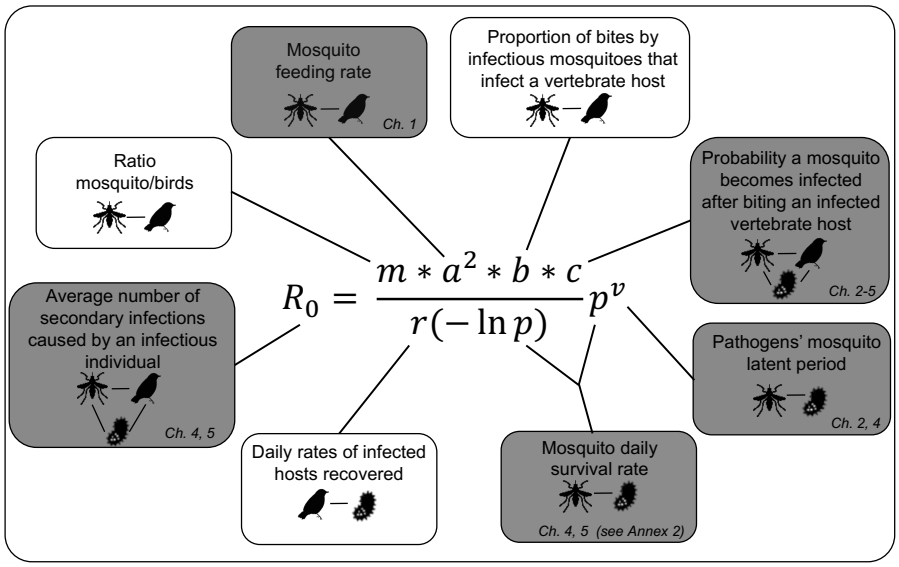

Fig 1. Adapted schematic representation of the Ross-Macdonald model to estimate R_0 for human malaria. This formula is applicable for other vector borne pathogens such as avian malaria or West Nile virus. Grey boxes with 'Ch.' followed by numbers represent the variables studied and the chapter/s where they are addressed. Figures in boxes ilustrate the interacting organisms (i.e. host, vector and pathogen) that affect each variable considered.

Mosquitoes have deserved great importance in medicine and veterinary sciences due to the important role they play in the transmission of numerous vector-borne pathogens to humans and animals (Beerntsen *et al.* 2010; see below). The blood-sucking behaviour of mosquitoes is a highly complex phenomenon that strongly affects the contact rate between infected and susceptible hosts. These interactions are driven by both host and vector related factors (Takken & Verhulst, 2013) (Fig. 2). Mosquitoes, like other blood-sucking insects, show preferences to feed on particular host species or individuals (Muñoz *et al.* 2012; Martínez-de la Puente *et al.* 2015) but these preferences may in turn be partially modulated by host-related factors such as abundance, anti-mosquito defensive behaviour or the emission of thermal and chemical cues (Takken & Verhulst, 2013). Altogether, these factors may have important consequences for the transmission success of parasites

(Kilpatrick *et al.* 2006; Takken & Verhulst, 2013) and are essential to identify the relative importance of each mosquito species as vector of these pathogens.

Following the ingestion, the development of the pathogen to complete its life cycle in the mosquito may differ depending on each group of pathogens, leading to complex interactions with the mosquito's midgut cells. For example, viruses usually come in contact with membrane proteins of the mosquito midgut cells (Hardy *et al.* 1983; Abraham & Jacobs-Lorena, 2004), while protozoans require the initial contact between male and female gametes to develop zygotes before coming in contact with proteins from the membrane of midgut cells to complete their life cycle (Valkiūnas, 2005). In spite of these differences, pathogens usually follow the same common steps; i.e. they are ingested, exposed to the environment of the middle intestine and the wall of the stomach and finally, reach the salivary glands for its transmission to a new vertebrate host (Hardy *et al.* 1983; Abraham & Jacobs-Lorena, 2004).

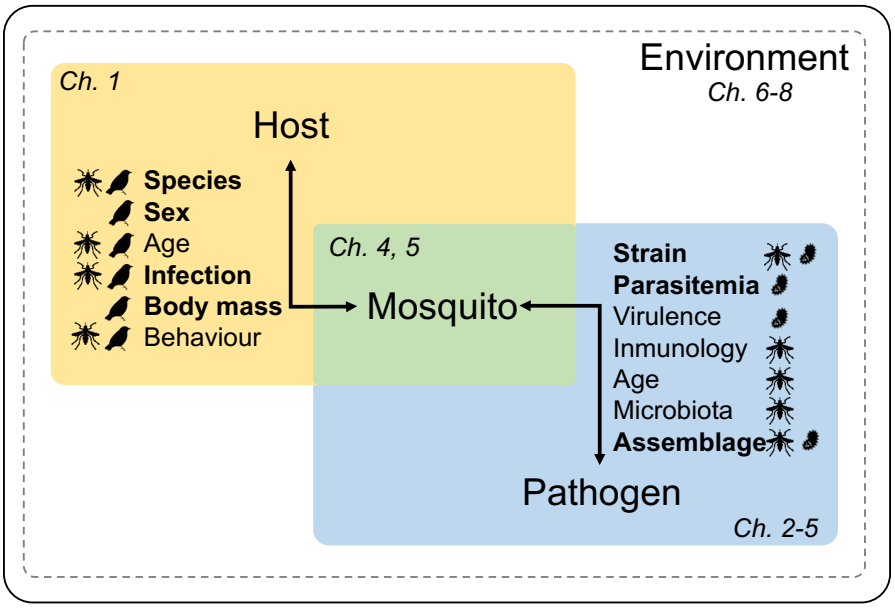

Fig 2. Schematic representation of the main factors affecting host-mosquito-pathogen interactions (solid lines). The organisms associated to the factors are illustrated with silhouettes. 'Ch.' followed by numbers indicates the chapter/s of the thesis in which these factors are studied.

Pathogens have to pass different barriers in the digestive system and the salivary glands of the mosquitoes that could interfere with its development, thus representing a major selective force for them (Smith *et al.* 2014). Therefore, to be successfully transmitted, vector-borne pathogens need to reach a competent vector to complete their life cycle. The ability of a mosquito to transmit a pathogen is frequently referred to as *vector capacity* or *vector competence*. Although both terms are indiscriminately used, some differences exist between them (BOX 1). However, for simplicity, I will use the term vector competence throughout the thesis to describe the ability of mosquitoes to transmit the studied pathogens.

BOX 1. Vector capacity *vs* vector competence

Vector capacity should be considered a broader term as it takes into account all factors that could influence the interactions between vectors, pathogens and vertebrate hosts, including environmental, behavioural, cellular, genetic and biochemical factors (Black *et al.* 1996; Woodring *et al.* 1996; Figure 1). By contrast, vector competence is governed by factors intrinsic to the mosquito, which influence the ability of a vector to transmit a pathogen (Hardy *et al.* 1983; Black *et al.* 1996; Woodring *et al.* 1996). Therefore, factors such as blood-sucking behaviour or the susceptibility of the mosquitoes to be infected by the pathogen, which have a strong genetic basis (Hardy *et al.* 1983; Woodring *et al.* 1996), may affect the vector competence.

The identification of factors determining the ability of pathogens to develop in different mosquito species remains an open question. The existence of specific mosquito-pathogen assemblages (Cohuet *et al.* 2010) often results in interspecific differences in the ability of the pathogens to develop in their vectors. In addition, strong controversy exists with respect to the costs of pathogen infections in the potential insect vectors. In this regard, it is important to highlight that vectors are also hosts for the pathogens and therefore, by definition, they are expected to suffer the costs of infections (see **Annex 2**). For example, the mosquito midgut is perforated when ookinetes of malaria parasites pass through, which might increase susceptibility to bacterial infections (Vaughan *et al.* 1996). In addition,

infected mosquitoes can mount a diverse array of immune responses when invaded by pathogens (Barillas-Mury *et al.* 2000), which might also affect mosquito survival (Ferguson & Read, 2002). However, all these factors affecting the interactions between pathogens and potential mosquito vectors have been traditionally neglected, especially for those pathogens affecting wildlife. The fact that some pathogens show a very low prevalence of infection in wild individuals finally limits the capacity to study these factors under field conditions. In addition, the study of these interactions requires biosafety laboratories (Level 2 or 3 depending on the risk that the pathogen may pose for humans and wildlife), which may limit these studies to those researchers with the ability to access these facilities. To partially solve these limitations, in addition to different flavivirus, I have used avian malaria parasites as the main study model in this thesis. Avian malaria has historically been considered an excellent model system for investigating the biology and transmission of *Plasmodium* parasites (Marzal, 2012).

Throughout the eight different chapters of this thesis, I assess how the interactions between vertebrate hosts, insect vectors, and pathogens affect the transmission of avian malaria parasites and *flaviviruses*, both transmitted by mosquitoes. In order to minimize the potential effects of artificial selection in the studied processes, no birds, mosquitoes, or avian malaria parasites from colonies maintained in laboratories were used during the course of this thesis.

The protagonists

The insect vectors

Mosquitoes are considered the main vectors involved in the transmission of many different pathogens (Becker *et al.* 2003). Viruses including WNV, Chikungunya, and Dengue, protozoans (malarial parasites), and nematodes (*Dirofilaria* worms) are all transmitted by mosquitoes (Tolle, 2009), supporting their relevance in studies of medical and veterinary entomology, as well as epidemiology and global health (Becker *et al.* 2003). Over 3500 species of mosquitoes have been described worldwide, 34 of them being recorded in Spain. In addition to native mosquito species, during the last decades, the invasive *Aedes aegypti* and *Aedes albopictus* (Asian tiger) have been recorded in the country (Bueno-Marí *et al.* 2012). *Aedes aegipty* was eradicated during the mid-20th

century, until its recent introduction on Fuerteventura (Canary Islands) from where it was also apparently eradicated in 2018. However, *Ae. albopictus* is spreading through the Mediterranean coast and rural areas of Spain, including Andalusia, Valencia Catalonia, Madrid, Navarra and Aragon (MSSSI, 2017). Additionally, *Ae. albopictus* has been recorded in the Basque Country and isolated areas of Portugal (Collantes *et al.* 2015; Osório *et al.* 2018). As in many studies assessing vector competence and patterns of transmission, only female mosquitoes were used here, as this is the only blood-sucking gender because they require blood proteins for egg laying. In this thesis, I focus on mosquito species with clear differences in their blood feeding patterns. While some species feed mainly on birds (e.g. *Culex pipiens*), others prefer to feed on mammals (e.g. *Aedes* (*Ochlerotatus*) *caspius*) or humans (e.g. *Aedes aegypti* or *Aedes albopictus*) (Muñoz *et al.* 2012; Martínez-de la Puente *et al.* 2015).

Hippoboscids (Diptera, Hippoboscidae), commonly known as louse flies, are common blood-feeding ectoparasites of mammals and birds (Hutson, 1984). This insect group includes more than 200 species distributed all over the world, of which about 60 have been cited in the Palaearctic and approximately 20 in Spain, 9 of them in the Canary Islands (Hjorth-Andersen Carlés Tolrá, 2002). Hippoboscids are competent vectors of Haemosporidian parasites, specifically *Haemoproteus* protozoans of the subgenus *Haemoproteus* (Valkiūnas, 2005; Levin *et al.* 2012; Santiago-Alarcon *et al.* 2012).

Pathogens

Avian Haemosporidians (phylum Apicomplexa) are a group of widespread blood parasites infecting birds that are transmitted by insect vectors (Valkiūnas, 2005). In addition to the avian malaria parasites of the genus *Plasmodium*, birds are commonly infected by the related malaria-like parasite *Haemoproteus*. The cycle of these parasites requires an insect vector for sexual reproduction and sporogonic stage, and a vertebrate host for asexual reproduction and merogony stage (Valkiūnas, 2005). When a mosquito feeds on an infected bird, parasite gametocytes develop to gametes in the insect midgut and fuse as zygotes to form ookinetes. After that, parasites penetrate into the midgut wall of mosquitoes, where ookinetes develop into oocysts to form sporozoites. Parasite sporozoites invade the salivary glands of the mosquito, located in their thorax. Parasites need approximately 7 to 22 days

to complete this cycle, depending on the parasite species (Valkiūnas, 2005; Palinauskas *et al.* 2016; Fig. 3). In the subsequent mosquito blood meal, sporozoites, the parasite infective form, are injected to the bloodstream of birds, where they develop asexual reproduction in internal organs, until the invasion of erythrocytes where parasites develop into gametocytes.

In addition to avian Haemoporidians, in this thesis I have also studied two strains of Zika virus (Flaviviridae). In 2015, the outbreak of Zika virus in Brazil spread into other countries of South and North America. Overall, Zika virus probably affected millions of people (Zanluca *et al.* 2015), although it is difficult to determine the exact number of cases as Zika infections usually course asymptomatic. Only in some cases, Zika virus infections shows symptoms such as fever or rash (Duffy *et al.* 2009), but more importantly, in pregnant women, Zika virus can cross the placentae barrier causing microcephaly and other brain anomalies in fetuses (Johansson *et al.* 2016). In addition, Zika infections can result in Guillain-barré Syndrome in adults (Cao-Lormeau *et al.* 2016).

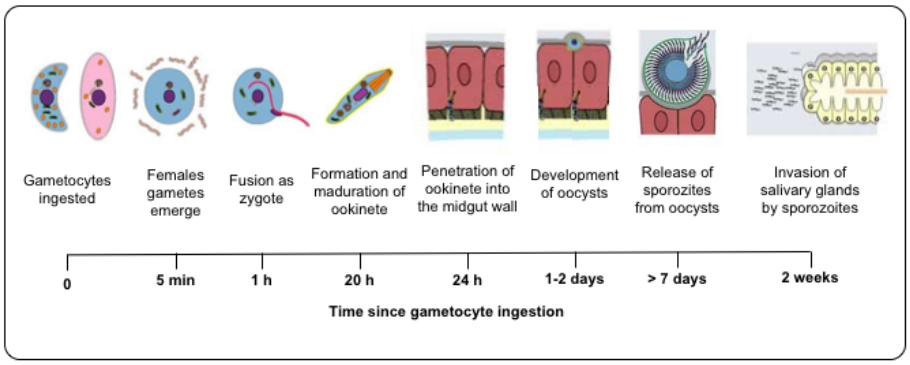

Fig 3. Schematic representation of the sexual life cycle of avian malaria parasites inside the mosquito (adapted from Baton & Ranford-Cartwright, 2005)**.**

Aedes aegypti plays a central role in the transmission of Zika virus (Li *et al.* 2012), but *Aedes alpobictus* may be involved in the occurrence of outbreaks as was the case of Gabon (Central Africa; Grard *et al.* 2014). Furthermore, sexual transmission of Zika virus has been documented in different countries, including Spain (Arsuaga *et al.* 2016). The geographical spread of mosquito vectors and the arrival of infected cases in areas with competent vectors, stress the necessity to perform further studies on the potential local

transmission of Zika virus, as may occur by autochthonous and invasive mosquito species present in Spain (Millet *et al.* 2017).

The vertebrate (avian) hosts

The widespread resident passerine house sparrow (*Passer domesticus*) is the main vertebrate study model used in this thesis. The body length of this species is about 14-18 cm and its body mass ranges from 21 to 31 g. Although body mass does not differ between sexes, adult house sparrows present strong sexual dimorphism in plumage coloration during the breeding season (Svenson *et al*. 2010). Additionally, jackdaws (*Corvus monedula*) were used in chapter 1 of this thesis. Jackdaws are non-migratory passerines, resident in Europe, Western Asia, and North Africa. This species is bigger than the house sparrow (about 34–39 cm long and a body mass ranging from 181 to 257 g) and does not present sexual dimorphism in plumage coloration (Svenson *et al.* 2010). Both house sparrows and jackdaws are commonly biten by mosquitoes (Muñoz *et al.* 2011) and are reservoirs and competent hosts of avian malaria parasites and related Haemosporidians (Marzal *et al.* 2008; Hellgren *et al.* 2009; Ferraguti *et al.* 2018) as well as WNV (Hamer *et al.* 2009; Loiseau *et al.* 2011; Pérez-Ramírez *et al.* 2014; Del Amo *et al.* 2014; Lim *et al.* 2014; Martínez-de la Puente *et al.* 2018).

In the third section of this thesis I have used the Eleonora's falcon (*Falco eleonorae*) as the model species to study the transmission dynamics of avian blood parasites in a wild bird population. The Eleonora's falcon is a long-distance migratory raptor that breeds throughout the Mediterranean basin and winters in Madagascar (Walter, 1979). This species exhibits a striking melanin-based colour polymorphism with individuals displaying a pale or dark morph with little variation within these two morphs (Gangoso *et al.* 2011). These two morphs show clear differences in the immune capacity (Gangoso *et al.* 2015) potentially affecting their interactions with pathogens.

Objectives

The main aim of this thesis is the study of the mechanisms of transmission of avian Haemosporidians and flaviviruses by mosquitoes, considering the two- and three-way interactions between insect vectors, vertebrate hosts, and pathogens.

First, I have studied some major factors affecting the interaction between mosquitoes and avian hosts. I have assessed the role of mosquito species and three host-related factors affecting the contact rates between mosquitoes and susceptible avian hosts in the transmission success of protozoans and arboviruses. In particular, I have assessed the effect of bird species, sex, body mass, and infection status by avian malaria parasites on the biting rate of two species of mosquitoes with different feeding preferences (section 1, chapter 1, objective 1).

Subsequently, in the second section of this thesis, I have studied how the interactions between mosquitoes and pathogens influence the transmission of pathogens. In chapter 2, I have studied the role played by alien and native mosquito species in the transmission of an emerging flavivirus (objective 2). I have estimated the vector competence for the ZIKV of two *Aedes* mosquitoes from Spain; the native *Aedes* (*Ochlerotatus*) *caspius* and the introduced *Aedes albopictus*. In chapter 3, I have assessed the competence of *Culex pipiens* for the transmission of *Plasmodium* and *Haemoproteus* parasites (objective 3). I have also investigated whether avian malaria parasites are locally adapted to their vectors (objective 4), by studying the competence of *Aedes* (*Ochlerotatus*) *caspius* and *Cx. pipiens* species from Southern Spain for the transmission of different *Plasmodium* lineages circulating in the area considering the impact of bird infection intensity and parasite identity on vector competence (chapter 4). Finally, to determine the effect of host infection intensity on parasite transmission risk (objective 5), I have experimentally determined the influence of *Plasmodium* load in the birds on parasite transmission risk (relative R_0) considering its effect on mosquito survival (chapter 5).

In the third section, I have conducted an observational study to determine the dynamics of transmission of avian malaria parasites and related Haemoporidians in a wild bird population. I have determined the relative importance of habitat-related *vs*. host-related mechanisms on parasite transmission (chapter 6, objective 6) and the phisiologic and genetic mecanisms that determine the infection in Eleonora's falcons (chapter 7, objective

7). Moreover, I have assessed how enviroment aspects could determine the infections (chapter 8, objective 8).

General methods

Study areas and sampling

The fieldwork for chapters 1 to 5 was performed in Andalusia (Southern Spain), an area characterized by a Mediterranean climate with long dry summers and low precipitation in winter. House sparrows and jackdaws were caught in the provinces of Huelva and Seville. Mosquito larvae were collected from surrounding areas where the birds were captured with the exception of *Ae. albopictus* included in chapter 2, which were collected in the metropolitan area of Barcelona (Northeast Spain).

Fieldwork was also conducted on Alegranza islet to accomplish chapters 6, 7 and 8. Alegranza (1050 ha, 289 m a.s.l.) is the northernmost island of the Canary Archipelago, located 100-km west off the African coast. It has a volcanic origin and the climate is semiarid, being particularly dry in summer, when strong easterly trade winds prevail. Adult and/or nestling Eleonora's falcons were mainly sampled on Alegranza, but also in their breeding colonies located on islands of the Mediterranean Sea (Andros (Greece), Kef Amor (Algeria), Sa Dragonera and Illa Grossa (Baleares Islands, Spain)) covering most of the species' breeding range. In addition, louse flies were collected from birds breeding on Alegranza.

Mosquito rearing and maintenance

The mosquito species used in the different chapters of this thesis were *Cx. pipiens*, *Ae. albopictus*, *Aedes aegypti* and *Ae.* (*Oc.*)*caspius*. The genus *Ochlerotatus* has been traditionally classified as an independent group, but a recent taxonomic study included *Ochlerotatus* as a subgenus of *Aedes* (Wilkerson *et al.* 2015). Immediately after collection, mosquito larvae were maintained under standard conditions (28 ± 1 ºC, 65–70% (RH) and 12:12 light: dark cycle). Emerged adult mosquitoes were anaesthetized, sexed and identified morphologically to species level. Female mosquitoes were starved for 24 hours before exposing them to birds or to an infected blood meal (see below) and were maintained in insect rearing cages.

Most studies included in this thesis were conducted using the facilities of the Estación Biológica de Doñana (EBD-CSIC), with the exception of the study of chapter 2. In this case, experimental procedures were done at the Biosafety Level 3 Laboratory of the

Wadsworth Center, Department of Health of the State of New York (Albany, USA). Two different fresh virus strains of ZIKV (Cambodia and Puerto Rico strains) were obtained from supernatant from infected C6/36 cultures harvested at 120 hours pi (MOI~1.0) and diluted 1:1 with blood-sucrose mixture without freezing. Mosquitoes were infected with a blood meal warmed to 37 ºC using a Hemotek membrane feeding with an intestine pig membrane as feeding system (Discovery Workshops Lancashire, United Kingdom) with fresh virus diluted 1:1 in defibrinated sheep blood (Colorado Serum Co.) with 2.5% sucrose. Additional, *Ae*.(*Oc.*) *caspius* were also inoculated by thorax microinjection (1ul) of supernatant of ZIKV infected C6/36 cultures.

Saliva isolation

One of the main methodological approaches used in this thesis is the use of mosquito saliva for the identification of vector-borne haematozoa. Since Hurlbut, (1966), mosquito saliva has been used to determine the presence of virus in studies on vector competence. However, this method has been rarely used for malarial parasites. In brief, this method consists in inserting the mosquito proboscis into 1 µL micropipette (microcaps®, Drummond Scientific Company, PA, USA) filled with 1 µL of foetal bovine serum (FBS) (Mores *et al.* 2007), although other different types of media such as oil or blood could be also employed. Additionally, 1µl of 2% pilocarpine, an analogue of the acetylcholine, could be applied on the mosquito thorax to stimulate salivation. After 45 min, the medium containing the mosquito saliva could be used as a source of viruses for subsequent replication (Nayar *et al.* 1980) or parasite DNA for further molecular detection or quantification (Fig. 4; Gutiérrez-López *et al.* 2016, **Chapter 3**).

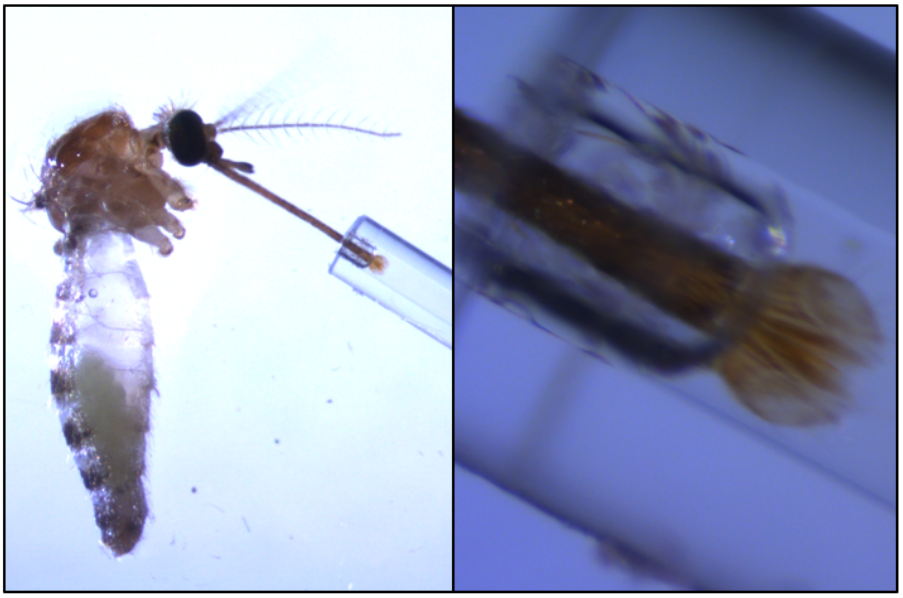

Fig 4. Pictures showing the collection of mosquito saliva samples.

Molecular analyses

Extraction of genomic DNA is an essential step in molecular analyses, as this may determine the subsequent success of DNA amplification and sequencing. After comparing the effectiveness of four DNA extraction methods (see **Annex 1**), in this thesis I isolated genomic DNA of blood samples from birds and the head-thorax of insects using the Maxwell®16 LEV system Research kit (Promega, Madison, WI). In addition, genomic DNA from mosquito saliva (and the parasites potentially present) was isolated using the DNeasy® Kit Tissue and Blood kit (Qiagen, Hilden, Germany). Parasite infections by avian Haemosporidians were identified following the broadly used protocol described by Hellgren *et al.* (2004). In addition, blood smears were used for the identification of parasites infecting birds and their quantification.

Section 1
Host-Vector Interactions

Chapter 1

Effects of host sex, body mass and infection by avian *Plasmodium* on the biting rate of two mosquito species with different feeding preferences.

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Abstract

The transmission of vector-borne pathogens is strongly influenced by the contact rates between vectors and susceptible hosts. The biting rates of mosquitoes depend on different factors including mosquito species and host-related traits (i.e. odour, heat and behaviour). However, host characteristics potentially affecting intraspecific differences in the biting rate of mosquitoes are poorly known. Here, we assessed the impact of three host-related traits (sex, body mass and infection status by the avian malaria parasite *Plasmodium*) on the biting rate of two mosquito species with different feeding preferences: the ornithophilic *Culex pipiens* and the mammophilic *Ochlerotatus caspius*. Seventy-two jackdaws *Corvus monedula* and 108 house sparrows *Passer domesticus* were immobilized and individually exposed to mosquito bites, to test the effect of host traits on biting rates. *Ochlerotatus caspius* showed significantly higher biting rates than *Cx. pipiens* on jackdaws, but non-significant differences were found on house sparrows. In addition, more *Oc. caspius* fed on female than on male jackdaws, while no differences were found for *Cx. pipiens*. The bird infection status and body mass of both avian hosts were not related to the biting rate of both mosquito species. Host sex was the only host-related trait potentially affecting the biting rate of mosquitoes, although its effect may differ between mosquito species.

Introduction

The blood-feeding behaviour of mosquitoes is a complex phenomenon that involves different steps. The initial seeking and location of hosts depends on the integration of chemical (CO_2, odours) and visual cues (e. g. host's size and plumage/pelage coloration) emitted by the host (Takken & Knols, 1999; Lehane, 2005). In close proximity between mosquitoes and their hosts, odour, heat and host defensive behaviour may affect the final host choice and blood-feeding success of mosquitoes (Raji & DeGennaro, 2017).

Under natural conditions, mosquitoes show different innate feeding preferences, with some mosquito species feeding mostly on mammals (mammophilic species, and some of them can be characterized as anthropophilic), while others preferring to bite on birds (ornithophilic species), or even amphibians or reptiles, yet other species show a more opportunistic behaviour (Molaei *et al.* 2017; Burkett-Cadena *et al.* 2008; Muñoz *et al.* 2012; Takken & Verhuls, 2013; Martínez-de la Puente *et al.* 2015). In addition to this broad tendency for particular host classes, mosquitoes bite certain host species at higher rates than those expected from their abundance (Kilpatrick *et al.* 2006; Hamer *et al.* 2009; Lura *et al.* 2012). For instance, Kilpatrick *et al.* (2006) showed that American robins (*Turdus migratorius*) were more intensely bitten by *Culex pipiens* mosquitoes than European starlings (*Sturnus vulgaris*) in North America. Similarly, in Europe, the feeding preference of *Cx. pipiens* for blackbirds (*Turdus merula*) was higher than for European starlings (Rizzoli *et al.* 2015). Within host species, some individuals may receive most mosquito bites and, as a result, they may play a role as superspreaders when infected by vector-borne pathogens (Liebman *et al.* 2014).

This heterogeneity in vector attraction and actual host use by mosquitoes could have an important impacts on transmission dynamics of parasites causing human and animal diseases (VanderWaal & Ezenwa, 2016). Therefore, factors affecting the contact rates between mosquitoes and susceptible vertebrate hosts largely determine the transmission success of vector-borne parasites such as protozoans (e.g. *Plasmodium*) and filarial worms (e.g. *Dirofiliaria spp.*) (Lehane, 2005; Dye, 1992).

Different non-mutually exclusive mechanisms may determine that an individual host receive more mosquito bites, such as the use of habitats with higher abundance of mosquitoes, higher emission of attractive thermal or chemical cues, or a less intense or

effective antimosquito behaviour than other individuals (Takken & Verhuls, 2013). In addition, hosts that are heavier (using body mass, as a correlate of body size), may receive more mosquito bites (Gillies & Wilkes, 1972) probably due to the higher amount of cues (e.g. CO_2) released by larger individuals (Torr *et al.* 2006). Different studies at the interspecific level have reported a positive relationship between host body mass and the feeding rate of different blood-sucking insects (Martínez-de la Puente *et al.* 2010; Schönenberger *et al.* 2016). However, very few studies have experimentally tested the relationship between species variation in body mass and the feeding rate of mosquitoes (Simpson *et al.* 2009). In addition, sex-specific morphological, physiological and/or behavioural characteristics could produce differences in the attraction of insect vectors (Zuk *et al.* 1990). These differences in vector attraction between host sexes have been argued as a potential explanation for the usually higher prevalence of blood parasites found in male than in female birds (Skorping & Jensen, 2004; Zuk & Stoehr, 2010; Calero-Riestra & García, 2016). To the best of our knowledge, only Burkett-Cadena *et al.* (2014) evaluated the effect of bird sex on the variation in mosquito biting preferences. By analysing the blood meal origin of mosquitoes, authors found that blood meals were biased towards male birds, but only in mammophilic mosquitoes. However, the reasons behind these differences remain unclear. The patterns found by Burkett-Cadena *et al.* (2014) could be the result of differential susceptibility and/or exposure of bird sexes to mosquito attacks or, simply, an unbalanced bird sex-ratio in the field. Finally, the host infection status by vector-borne parasites may also influence the mosquito biting patterns, potentially determining the pathogen transmission success (Cornet *et al.* 2013). For example, humans infected by *Plasmodium vivax* were more attractive to mosquito vectors (Batista *et al.* 2014). However, studies with avian *Plasmodium* are less conclusive, because *Culex pipiens*, the main vector of avian *Plasmodium*, was reported to preferentially bite chronically infected birds over uninfected individuals according to Cornet *et al.* (2013), while the opposite pattern has also also been reported (Lalubin *et al.* 2012), and even the absence of significant differences between infected and uninfected birds (Yan *et al.* 2017).

In this study, we experimentally assessed the impact of three host related traits (bird body mass, sex and infection status by the avian *Plasmodium spp.*) on mosquito feeding patterns, while removing host antimosquito behaviour. We performed this study using two

potential avian malaria mosquito vectors with different feeding preferences: the ornitophilic *Culex pipiens* and the mammophilic *Ochlerotatus* (*Aedes*) *caspius* (Martínez-de la Puente *et al.* 2015; Santiago-Alarcon *et al.* 2012; Ferraguti *et al.* 2013). We used two bird species as host models, the jackdaw (*Corvus monedula*) and the house sparrow (*Passer domesticus*). Both bird species are common hosts of avian malaria parasites (Hellgren *et al.* 2009; Drovetski *et al.* 2014). Based on previous evidence (Schönenberger *et al.* 2016; Burkett-Cadena *et al.* 2014; Cornet *et al.* 2013), we predict, i) a higher biting rate on birds in the ornithophilic *Cx. pipiens* than in the mammophilic *Oc. caspius*, ii) a higher mosquito biting rate on heavier individuals, iii) a higher biting rate on male birds over females, especially for *Oc. caspius*, and iv) a higher biting rate on *Plasmodium* infected birds than on uninfected individuals.

Material and methods

Mosquito collection and rearing

Culex pipiens and *Oc. caspius* larvae were collected in the wild 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), respectively. Larvae were transferred to the laboratory and kept in plastic trays with fresh or brackish water, respectively, and fed *ad libitum* with Mikrozell 20ml/22g (Dohse Aquaristik GmbH & Co.101 KG, D-53501, Gelsdorf, Germany). Larvae and adult mosquitoes were maintained at standard conditions (28 ± 1 ºC, 65–70% (RH) and 12:12 light: dark cycle). Adult mosquitoes were anesthetized with ether and their sex and species identified based on morphology, on chilled Petri dishes using a stereomicroscope (Nikon SMZ645) following Schaffner *et al.* (2001). After identification, adult females were placed in insect cages (BugDorm-43030F, 32.5×32.5×32.5 cm) and fed *ad libitum* with 1% sugar solution. 24h prior to each experiment, female mosquitoes were deprived from sugar solution. Laboratory maintained colonies of mosquitoes were not used to minimize the effects of artificial selection of mosquitoes with particular biting preferences (Franks *et al.* 2011; Lagisz *et al.* 2011).

Bird sampling and experimental procedure

The jackdaw is a non-migratory passerine bird, resident in Europe, western Asia and North Africa. It is 34–39 cm long and its body mass ranges from 181 to 257 g. This species is not sexually dimorphic. The house sparrow is also a non-migratory passerine, native to most Europe. It is 14-18 cm long and its body mass ranges from 21 to 31 g. Although body mass does not differ between sexes, adults of this species present strong sexual dimorphism in plumage coloration (Svensson *et al.* 2010).

The jackdaws were caught from May to September 2014 in 'La Cañada de los Pájaros' using a walk in trap and the house sparrows were caught using mist nets from April to June 2014 in the same location, and from June to September 2016 in different localities from the Huelva province. Birds were individually ringed with numbered metal rings weighed and blood was sampled from the jugular vein using sterile syringes. The volume of blood obtained differed between species due to differences in body mass (i.e.1 ml in jackdaws and 0.2 ml in house sparrows). Female birds with brood patches were released immediately after capture and were not included in this study to reduce any impact on their reproductive performance. The experimental feeding trials were developed from April to September 2014 and 2016 and from 7:30 am to 12:00 am (GMT +1hour).

Individual birds were enclosed for 30 minutes in an insect cage (BugDorm-43030F, 32.5×32.5×32.5 cm) containing 54 ± 33.7 (mean±SD) (Range 1-152) mosquito females of either *Cx. pipiens* or *Oc. caspius*. The experimental feeding trials were developed in an environment with low light and no noise that could alter their behaviour. A number of previous studies have reported the ability of mosquito species including *Cx. pipiens* to feed on birds maintained in cages (Burkett-Cadena *et al.* 2010; Gutiérrez-López *et al.* 2016). Each bird was immobilized to prevent defensive behaviours against mosquitoes. Jackdaws were immobilized using non-permanent masking tape, with the wings attached to the body, the beak closed and legs held together. Un-feathered areas of the body (i.e. legs and eyes) remained uncovered during the trials, thus allowing mosquitoes to feed on the birds. House sparrows were immobilized using a cylinder made with 1×1 cm mesh, allowing mosquitoes to bite through. After the trials, birds were released at the same location of capture without any apparent sign of damage. Mosquitoes with a recent blood meal in their abdomen were counted and stored in Eppendorf tubes.

All experimental procedures were approved by the CSIC Ethics committee and Animal Health authorities (439-2016), and complied with Spanish laws.

Molecular analyses

Genomic DNA was extracted from blood samples using the MAXWELL® 16 LEV Blood DNA Kit (Gutiérrez-López *et al.* 2015). Birds were molecularly sexed following Griffiths *et al.* (1998). The *Plasmodium* infection status of birds was assessed by the amplification of a 478-bp fragment of the mitochondrial cytochrome b gene following Hellgren *et al.* (2004). The presence of amplicons was verified in 1.8% agarose gels and positive samples were sequenced using the BigDye technology (Applied Biosystems) or the Macrogen sequencing service (Macrogen Inc., Amsterdam, The Netherlands). Sequences were edited using the software Sequencher™ v 4.9 (Gene Codes Corp., © 1991–2009, Ann Arbor, MI 48108) and assigned to parasite genus after comparison with the GenBank database (National Centre for Biotechnology Information).

Statistical analysis

The proportion of mosquitoes that bit house sparrows and jackdaws were compared separately for the two mosquito species using Chi-Square tests. We used Generalized Mixed Linear Models (GLMMs) with binomial error and logit link function to assess the effect of mosquito species and bird characteristics on mosquito biting rates. Analyses were performed in R software 3.2.5 (R Core Development Team, 2016) with the package *lme4* (Bates *et al.* 2015). First, we compared the biting rates of the two mosquito species on birds. Models included the mosquito biting rate as the dependent variable, expressed as the number of mosquitoes that bit on the focal bird with respect to the number of mosquitoes that did not bite this individual using the *cbind* function. Due to the differences in the method used to immobilize each bird species and their clear differences in body size, separated models were fitted for jackdaws and house sparrows. In each case, mosquito biting rate (expressed as reported above) was included as the dependent variable, bird body mass as a covariate and bird sex, *Plasmodium* infection status (infected/uninfected) and mosquito species (*Cx. pipiens*/*Oc. caspius*) as fixed factors. We also included the interaction between mosquito species and host sex and between mosquito species and

infection status in the models. Bird identity was included as a random term to correct for the overdispersion shown when using both binomial and quasibinomial distributions (dispersion parameter > 7.21) (Harrison 2014). Body mass was scaled for each species by the standar deviation and mean-centred to normalize the variable distribution. The jackdaw population studied here was subjected to a medication experiment with birds either injected immediately before exposure to mosquitoes with a sub-curative dose of primaquine or treated as controls. This treatment did not affect the mosquito-biting rate (Z = -1.2, est = -0.62, P = 0.26), thus this factor was not included in further analyses.

Results

Seventy-two jackdaws (34 males and 38 females) and 108 house sparrows (71 males and 37 females) were included in this study. Of them, thirty jackdaws (41.7%) and sixty-one house sparrows (56.5%) were infected by avian *Plasmodium*. A total of 9601 mosquito females were exposed to the birds, including 6836 *Cx. pipiens* and 2765 *Oc. caspius*. Of them, 656 (9.6%) *Cx. pipiens* and 633 (22.9%) *Oc. caspius* fed on birds (Table 1), including 294 (44.8%) *Cx. pipiens* and 436 (68.9%) *Oc. caspius* feeding on jackdaws and 362 (55.2%) *Cx. pipiens* and 197 (31.1%) *Oc. caspius* feeding on house sparrows (Table 1).

The biting rate of *Oc. caspius* on jackdaws was higher than on house sparrows (Chi-square = 361.18, d.f. = 1, P <0.001), while no differences were found for *Cx. pipiens* (Chi-square = 0.72, d.f. = 1, P = 0.39; Fig. 1). The mammophilic *Oc. caspius* showed a significantly higher biting rate than the ornithophilic *Cx. pipiens* (Z = 6.09, est = 2.20, P <0.001; Fig. 1).

The effects of bird traits on mosquito biting rates were studied separately for each bird species because the differential methodology used in each species and the differences reported above. *Oc. caspius* showed a significantly higher biting rate than *Cx. pipiens* in jackdaws (Table 2)

Table 1. Summary data of mosquitoes biting jackdaws and house sparrows used in this study with respect to host sex and infection status by avian *Plasmodium* parasites.

Jackdaws			N	Mean of mosquitoes in each assay per box (SE)	Mean of engorged mosquitoes per box (SE)
Cx. pipiens					
	Sex	Male	26	59.1 (6.5)	7.0 (2.6)
		Female	29	49.7 (6.2)	3.6 (1.0)
	Infectious status	Uninfected	32	58.7 (5.9)	4.9 (2.9)
		Infected	23	47.8 (7.1)	6.0 (3.5)
Oc. caspius					
	Sex	Male	8	62.9 (12.0)	17.0 (4.8)
		Female	9	58.1 (11.2)	33.5 (6.2)
	Infectious status	Uninfected	10	71.9 (10.7)	21.3 (5.8)
		Infected	7	45.9 (12.8)	30.3 (7.3)
House sparrows			N	Mean of mosquitoes in each assay per box (SE)	Mean of engorged mosquitoes per box (SE)
Cx. pipiens					
	Sex	Male	47	57.9 (4.9)	4.8 (0.9)
		Female	21	54.2 (7.6)	6.5 (1.3)
	Infectious status	Uninfected	25	52.0 (6.8)	4.6 (1.2)
		Infected	43	59.5 (7.3)	5.8 (1.2)
Oc. caspius					
	Sex	Male	24	43.8 (7.0)	4.3 (1.2)
		Female	16	42.8 (8.8)	5.8 (1.5)
	Infectious status	Uninfected	22	33.9 (7.3)	3.5 (1.3)
		Infected	18	54.9 (8.3)	6.7 (1.4)

In addition, *Oc. caspius* showed a higher biting rate on female than on male jackdaws, while non-statistically significant differences were found for *Cx. pipiens* (Table 2, Fig. 1). The variables host infection status by avian *Plasmodium* and body mass were not significantly related to mosquito biting rates (Table 2). For house sparrows, we found no differences in the biting rate between *Cx. pipiens* and *Oc. caspius*. Host sex, body mass and infectious status by avian *Plasmodium* were not significantly related to mosquito-biting rates (Table 2, Fig. 1).

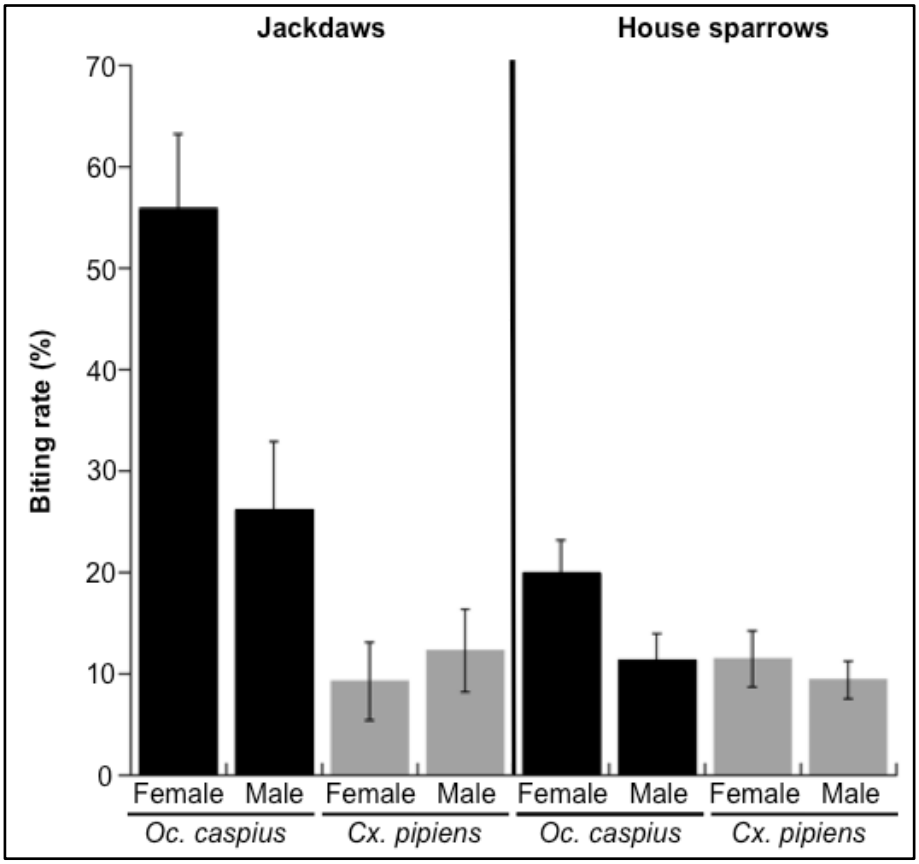

Fig 1. Biting rate (%) of *Ochlerotatus caspius* and *Culex pipiens* mosquitoes on female and male jackdaws and house sparrows.

Table 2. Results of GLMMs analysing mosquito-biting rates in relation to mosquito species (*Ochlerotatus caspius* and *Culex pipiens*) and birds' body mass, sex and *Plasmodium* infection status. The interactions between variables are indicated with *. Significant effects are highlighted in bold.

	Jackdaws				House sparrows			
Explanatory variables	est	std. Error	Z	*P*	est	std. Error	Z	*P*
Mosquito species	2.93	0.47	6.26	**<0.001**	0.33	0.54	0.62	0.54
Body mass	0.22	0.18	1.30	0.21	-0.02	0.13	-0.14	0.89
Sex	0.01	0.40	0.03	0.98	-0.44	0.35	-1.24	0.21
Infection status	0.28	0.31	0.92	0.36	0.08	0.35	0.25	0.81
Mosquito species*sex	-1.53	0.68	-2.26	0.02	-0.03	0.56	-0.05	0.96
Mosquito species*infection status	0.72	0.70	1.04	0.30	0.23	0.55	0.41	0.68
Explained variance (R2)	0.20				0.02			

Discussion

Identifying the potential causes underlying the non-random biting patterns of mosquitoes is essential to understand the dynamics of transmission of avian *Plasmodium* and other vector-borne pathogens in the wild (Liebman *et al.* 2014). Here, we tested how three important avian traits (i.e. body mass, sex, and the infection status by *Plasmodium*) in two bird species affect the biting rates by two mosquito species potentially involved in the transmission of avian malaria parasites (Ferraguti *et al.* 2013).

The biting rate of the mammophilic *Oc. caspius* on jackdaws was higher than the biting rate of the ornithophilic *Cx. pipiens*, while non-significant differences were found when mosquitoes faced house sparrows. Although most of the blood meals of *Oc. caspius* analysed in different studies derived from mammals, birds including chickens and house sparrows represent between 9.1% and 19.9% of the blood meals in this species (Muñoz *et al.* 2012; Balenghien *et al.* 2006). This pattern clearly contrasts with that of *Cx. pipiens,* for which birds represent between 85.1% to 91.67% of the bloodmeals (Muñoz *et al.* 2012). Our results clearly support the ability of *Oc. caspius* to feed on birds, at least when they are not allowed to choose between other host classes (i.e. mammals). This result is especially relevant, as our study focused on the biting rate of mosquitoes and not on the feeding preferences of these species. Contrary to *Cx. pipiens*, *Oc. caspius* is traditionally considered as an aggressive mosquito producing important nuisance in human populations (Gutsevich *et al.* 1974), but experimental studies supporting this assumption are scarce. Differences in the biting rate between mosquito species could be associated with their life history traits and breeding requirements, especially those related to the availability of water sources. While *Oc. caspius* depends on tidal cycles and use temporal flooded areas for larva development (Ezanno *et al.* 2015), *Cx. pipiens* use more permanent water sources (Roiz *et al.* 2014) and, consequently, their life cycle may be less time constrained. In addition, it is possible that a differential activity pattern between mosquito species could affect our results, with *Oc. caspius* showing a strong peak of activity during the day while *Cx. pipiens* peak its activity at night and sunset (Balenghien *et al.* 2006). Although this possibility could potentially explain the differences found between species, the biting rates of *Cx.*

pipiens found in this study are similar to those found in a previous experiment developed during the night (Yan *et al.* 2017).

The fact that differences in biting patterns were only detected when mosquitoes faced jackdaws, the larger host species, suggests that these may be related to differences in the amounts of cues emitted by each bird species. In close proximity to their hosts, the relevance of visual and thermo-sensory stimulation of mosquitoes increases with respect to larger distances. Moreover, the use of multiple sensory cues may increase the likelihood of mosquito feeding success (Raji & DeGennaro, 2017). Due to their larger size, jackdaws may emit a higher amount of attractants, including CO_2, heat, and odours than house sparrows, potentially leading to the differences found here.

Previous studies have reported a positive relationship between host body mass and biting rates of blood sucking insects (Schönenberger *et al.* 2016; Estep *et al.* 2012), although, this pattern usually correspond to studies comparing different host species. As expected from its larger size, jackdaws were bitten at a higher rate than house sparrows by *Oc. caspius*, but no differences were found for *Cx. pipiens*. Additionally, we did not find any significant relationship between the biting rate of mosquitoes and bird body mass at the intraspecific level. In this regard, Lalubin *et al.* (Lalubin *et al.* 2012) found that the attraction of *Cx. pipiens* to house sparrows was not significantly associated with their body mass. This suggests that, at short distances, the slight intraspecific differences in body mass are probably less important than other cues determining mosquito bites, like heat, humidity or odour (Raji & DeGennaro 2017).

Hosts'sex influenced the biting rates of *Oc. caspius* mosquitoes when facing jackdaws. This mosquito species preferred to bite female than male jackdaws, but these differences were not found when mosquitoes were exposed to house sparrows. The biting rates of *Cx. pipiens* were not significantly related to host sex. Burkett-Cadena *et al.* (Burkett-Cadena *et al.* 2014) found male-biased blood meals in mosquitoes (64% of the bloodmeals analysed derived from male birds), although they suggested that this could be due to skewed sex ratios in wild birds. However, no significant sex-biased differences were found in the feeding patterns of bird-biting mosquitoes, including *Culex* species (Burkett-*Cadena et al.* 2014). Moreover, Simpson *et al.* (2009) concluded that bird sex has no effect on the probability of *Cx. pipiens* to choose an individual over its partner. The preference of

mammophilic mosquitoes for a particular sex of bird could be associated with the sexual differences in the composition of odour profiles. Among other factors, the volatile and non-volatile substances of the secretions of the preen gland may affect the feeding preferences of blood sucking insects (Russell & Hunter, 2005; Martínez-de La Puente *et al.* 2011) and their composition differ between bird sexes (Jacob *et al.* 1979; Amo *et al.* 2012). Differences in the response of *Oc. caspius* and *Cx. pipiens* to secretions of the preen gland could explain, at least in part, discrepancies found between mosquito species (Allan *et al.* 2006).

We did not find support for a relationship between avian *Plasmodium* infection status and mosquito-biting rates. In view of previously reported results, it is unclear whether avian malaria infection enhances (Cornet *et al.* 2013) or decreases (Lalubin *et al.* 2012) the mosquito attraction towards the infected host. Thus, the host manipulation hypothesis pointing to an increase in *Plasmodium* transmission success through a higher attractiveness of infected host to mosquito bites remains an open question (Hurd, 2003; Lefèvre *et al.* 2009). Differences in the experimental procedure used, as well as in the intensity of infection between infected birds and/or the parasite and mosquito species studied could potentially explain these discrepancies. The results of our study should be interpreted with caution, as the host individuals used in our experiments were naturally infected by different lineages of *Plasmodium* and probably were in different stages of infection, which may affect host attractiveness for vectors. In addition to changes in the amount and quality of cues emitted by infected hosts, differences in the intensity of defensive behaviour associated with the infection status might explain differences in their susceptibility to mosquito attacks (Day *et al.* 1983; Shirasu & Touhara, 2011). For example, Day *et al.* (1983) found that malaria infected mice were more lethargic and less likely to defend against mosquitoes. In our study, birds did not show symptoms of lethargy and all were immobilized to prevent anti-mosquito defensive behaviour. Therefore, the possibility of changes in host defensive behaviour owing to *Plasmodium* infection was ruled out in this study. Additionally, it is possible that the potential differences in the emission of cues between infected and uninfected hosts (Kelly *et al.* 2015) can only be appreciated by mosquitoes at large distances, when host-seeking behaviour is mainly based on olfactory

clues (Raji & DeGennaro, 2017) or may be only evidenced when performing dual-choice experiments (Cornet *et al.* 2013), which is not the case in our study.

Conclusions

Our study highlights that the magnitude and direction of the effects of hosts' traits such as body mass, sex or the infection status by the mosquito-borne avian *Plasmodium* on the feeding patterns of mosquitoes are far from being generalizable. Only sex was associated to differences in mosquito biting rates, and this effect was only detected for one of the mosquito species studied here. Consequently, the biting patterns of mosquitoes may differ according to vector and host species characteristics. The reasons underlying the preference of mammophilic mosquitoes for individuals of a particular sex are unclear and need detailed analyses with regard, for instance, to the olfactory cues released by male and female birds.

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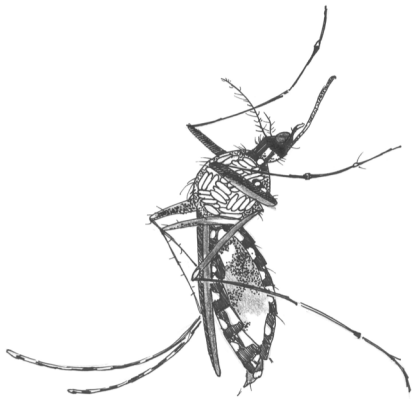
Section 2
Vector-Pathogen Interactions



Chapter 2

Vector competence for Zika virus of the native *Aedes caspius* and the invasive *Aedes albopictus* mosquitoes from Spain

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(*Under review*)

Abstract

Zika virus (ZIKV) is mainly transmitted by *Aedes aegypti*, although *Ae. albopictus* can also transmit ZIKV. However, the vector competence may vary geographically, and the competence of most European mosquitoes for ZIKV is currently unknown. We experimentally assessed the vector competence of *Ae. albopictus* and *Ae. caspius* from Spain for ZIKV strains Puerto Rico and Cambodia, and compared with *Ae. Aegypti*. We also determined the ability of *Ae. albopictus* to vertically transmit ZIKV. *Aedes albopictus* was a competent vector for ZIKV, with similar transmission rates to *Ae. aegypti* for both strains. However, ZIKV was unable to be transmitted by *Ae. caspius*. Moreover, ZIKV was vertically transmitted to *Ae. albopictus* progeny. This study highlights the potential of *Ae. albopictus* from Spain to transmit ZIKV and suggest the possibility that ZIKV could be transmitted locally in Spain. This is not the case of the non-competent vector for ZIKV *Ae. caspius*.

Introduction

Zika virus (ZIKV; *Flaviviridae*, *flavivirus*) is an emerging arbovirus associated with Guillain-Barré Syndrome and microcephaly in fetuses (Musso and Gubler, 2016). In 2015-16, the virus spread through the Americas causing an important outbreak (Faria *et al,*. 2016). Up to know, *Aedes aegypti* has been the main vector of most ZIKV epidemics (Li *et al,*. 2012; Calvez *et al.* 2016), although *Ae. albopictus* caused an outbreak in Gabon, and *Aedes polynesiensis* in French Polynesia (Grand *et al.* 2014; ECDC, 2014a). Moreover, various *Aedes* spp. have been suggested as potential vectors of ZIKV in laboratory-based studies (Gendernalik *et al.* 2017; Ledermann *et al.* 2014).

Aedes aegypti is absent from most European countries (ECDC, 2014b), but the invasive *Ae. albopictus* and other related native species could create novel epidemiological scenarios for ZIKV transmission. Indeed, *Ae. albopictus* populations from France, Italy and Germany are competent vectors for ZIKV (Jupille *et al,*. 2016; Di Luca *et al.* 2016; Heitmann *et al.* 2017), while *Culex pipiens* and *Cx. torrentium* from Germany are unable to transmit the virus (Heitmann *et al.* 2017). However, the vector competence for the transmission of ZIKV in most European mosquito species is currently unknown and may vary between virus strains and mosquito populations, which suggest that particular vector-virus assemblages may be more efficient for the virus transmission than others (Ciota *et al.* 2017).

Since July 2015, over 2,130 imported ZIKV cases have been confirmed in Europe (ECDC, 2014), although no autochthonous vector-borne transmission has been reported yet. In Spain, 316 ZIKV cases were confirmed in 2016 (MSSSI, 2017), 48 of them occurred in Barcelona (González *et al.* 2017), being all of them imported mainly from travelers coming from America and Asia. In this regard, a previous study addressing the risk of ZIKV introduction in Europe showed that southern Europe and eastern Spain have a high probability of ZIKV outbreak via travellers from America, also fuelled by the presence of *Ae. albopictus* in these regions (Rocklöv *et al.* 2016). *Aedes albopictus* has recently spread through the Mediterranean coast of Spain (Collantes *et al.* 2015), being particularly abundant in the metropolitan area of Barcelona. Another species of the genus *Aedes,* which is particularly abundant in Spain, is the marshland mosquito *Aedes* (*Ochlerotatus*) *caspius*

(Wilkerson *et al.* 2015). Under this scenario, it becomes essential to assess the actual role of these mosquito species in the transmission of ZIKV in Spain.

Here, we experimentally assessed the competence of Spanish populations of *Ae. caspius* and *Ae. albopictus* for the transmission of two ZIKV strains as compared with the competence of a colonized population of *Ae. aegypti.* In addition, we determined the ability of *Ae. albopictus* to vertically transmit ZIKV.

Material and methods

In 2016, *Ae. caspius* larvae and *Ae. albopictus* eggs were collected in Huelva (Southwestern Spain) and Barcelona (Northeastern Spain), respectively. Samples were shipped to the New York State Department of Health (NYSDOH) Arbovirus laboratory for their rearing (*Ae. caspius*) and colonization (*Ae. albopictus*). *Aedes aegypti* (provided by Gregory Ebel, Colorado State University) were originally collected in Poza Rica (Mexico) and colonized in the same facility. F1 generation of *Ae. caspius* was used in the experiments due to the inability to mate this species under laboratory conditions. F2 and F8 generations were used for *Ae. albopictus and Ae. aegypti*, respectively. Mosquitoes were reared and maintained in 30.5 cm^3 cages at 27°C, 50-65% relative humidity and 16:8 light:dark cycle.

ZIKV CAM (strain FSS130325, GenBank Accession # JN860885) was originally isolated in 2010 from human serum in Cambodia, and ZIKV PR (strain PRCABC59, GenBank Accession # KU501215) was isolated in 2015 from a patient serum infected in Puerto Rico (Ciota *et al.* 2017).

Four to 10 days old *Ae. caspius* females were exposed to ZIKV PR in three independent trials using different ZIKV concentrations (Table 1). Four to 7 days old *Ae. albopictus* and *Ae. aegypti* females were exposed to ZIKV CAM or ZIKV PR in an independent trial. All mosquitoes were offered blood meals for 2 hours at 37 ºC using a Hemotek feeding system (Discovery Workshops Lancashire, UK). Blood meals were prepared using ZIKV freshly propagated in C6/36 cells for 4 days and diluted 1:1 in defibrinated sheep blood (Colorado Serum Co. USA) with 2.5% sucrose (Ciota *et al.* 2017). Additionally, 16 unengorged *Ae. caspius* females were intrathoracically injected with 1µl of ZIKV PR stock at 5 $\log_{10}$ plaque forming units (PFU/ml).

After blood feeding, mosquitoes were sedated with CO_2 and engorged mosquitoes were transferred to 0.6 L paper cartons and provided cotton pads with 10% sucrose *ad libitum* and maintained at 27°C for experimental testing. Infection, dissemination and transmission rates of ZIKV were quantified at 7, 14 and 21 days post-infection (dpi) for *Ae. albopictus* and *Ae. caspius*, and also at 5 and 9 dpi for *Ae. aegypti* (Ebel *et al.* 2005). Infection and transmission rates for *Ae. caspius* infected by intrathoracic injection were evaluated at 9 dpi. For each day and mosquito species, 14-40 mosquitoes were sedated, and then, its legs removed and placed into 1 ml mosquito diluent [MD; 20% heat-inactivated fetal bovine serum (FBS) in Dulbecco's phosphate-buffered saline (PBS) plus 50 µg/ml penicillin/streptomycin, 50 µg/ml gentamicin, and 2.5 µg/ml Fungizone]. Mosquitoes were allowed to expectorate for 30 minutes into capillary tubes charged with ~20ul FBS plus 50% sucrose (1:1), and the mixture was placed into 250ul MD. Mosquito bodies were then placed in individual tubes with MD. All samples were stored at -80°C until analyses were performed.

To assess vertical transmission of the virus, 4-7 days old *Ae. albopictus* females (n = 200) were orally infected with ZIKV PR as described above. Additional non-infectious blood meals were offered weekly after the first oviposition. Eggs from the second oviposition were hatched and reared. Second instar larvae were grouped in pools of 5 individuals and tested for ZIKV. Vertical transmission rate, measured as filial infection rate (FIR), was estimated using a maximum likelihood estimate based on pool size and the number of positive pools for ZIKV per 1,000 larvae (PoolInfRate 4.0; Centers for Disease Control and Prevention, Atlanta, GA, USA).

ZIKV was quantified in bodies, legs and salivary secretion by qRT-PCR using the primers ZIKV 1086 and ZIKV 1162 (Lanciotti *et al.* 2008) to test infection, dissemination and transmission, respectively. ZIKV titers were calculated from standard curves based on infectious particle standards created from matched virus stocks.

We performed three similar Generalized Linear Models (GLMs) with binomial error distribution and logit link function to assess the effect of mosquito species, virus strains (fixed factors) and dpi (covariate) on the infection, dissemination and transmission rates. We also included the interactions between virus strain and dpi and between virus strain and mosquito species in the models. Differences in mean viral titers between

mosquito species, virus strains, and dpi in mosquito body, legs and saliva secretions were determined using the Kruskal-Wallis test. Analyses were run in JMP software v9 (SAS Institute, Cary, NC).

Results

ZIKV infection was detected in *Ae. caspius* at 7, 14 and 21 dpi (Table 1). However, ZIKV was not disseminated or transmitted by *Ae. caspius*. Mean viral titers in *Ae. caspius'* bodies were significantly lower than in *Ae. aegypti* and *Ae. albopictus* ($\chi^2 = 31.77$, d.f. = 2, $p < 0.001$). Overall, 87.5% of intrathoracically inoculated *Ae. caspius* were positive at 9 dpi, but only 14.3% of them had positive saliva secretion.

The infection rate differed between mosquito species and viral strains (Table 2), being higher in *Ae. albopictus* than in *Ae. aegypti*, and higher for ZIKV PR than for ZIKV CAM (Table 1). The dissemination rate increased with dpi, but no differences were found between mosquito species or ZIKV strains (Table 2). The transmission rate also increased with dpi (Table 2), but in this case, it differed between strains (Table 2), being higher for ZIKV CAM than for ZIKV PR (Table 1). Transmission of ZIKV CAM was observed from 7 dpi in both mosquito species, but transmission of ZIKV PR did not occur in *Ae. aegypti* and *Ae. albopictus* until 9 and 21 dpi, respectively (Tables 1 and 2).

The mean viral titers in bodies differed between mosquito species and ZIKV strains. Mean viral titers were higher in *Ae. albopictus* than in *Ae. aegypti* ($\chi^2 = 5.09$, d.f. = 1, $p < 0.02$) and mean viral titers of ZIKV PR were higher than those of ZIKV CAM ($\chi^2 = 6.92$, d.f. = 1, $p < 0.009$). Mean viral titers in legs were similar for both ZIKV strains ($\chi^2 = 0.95$, d.f. = 1, $p = 0.33$), although higher in *Ae. aegypti* than in *Ae. albopictus* ($\chi^2 = 9.53$, d.f. = 1, $p < 0.002$). No significant differences in mean viral titers of saliva secretions were found between mosquito species ($\chi^2 = 1.7$, d.f. = 1, $p = 0.19$) or ZIKV strains ($\chi^2 = 1.02$, d.f. = 1, $p = 0.31$) (Fig. 1).

Five of 17 (29.4%) F1 larval pools of *Ae. albopictus* were positive for ZIKV PR, with a FIR of 72.2 (95% CI: 27.6-156.1) and mean viral load of 2.5 $\log_{10}$ (PFU/ml).

Table 1. Infection, dissemination and transmission rates of mosquitoes experimentally infected with ZIKV CAM and ZIKV PR. The number of mosquitoes analyzed is shown in brackets.

Days post infection	Mosquito species	ZIKV strain	Blood meal titers (Log10 (PFU/ml))	% Infected	% Infected Disseminating	% Infected Transmitting
5	*Ae. aegypti*	CAM	7.6	27.3 (33)	11.1	0
		PR	7.6	37.5 (30)	25	0
7	*Ae. aegypti*	CAM	7.6	24.2 (33)	75	12.5
		PR	7.6	61.8 (34)	38.1	0
	Ae. albopictus	CAM	7.6	90.5 (21)	42	10.5
		PR	7.6	97.0 (33)	31.3	0
	Ae. caspius	PR	7.7	21.4 (14)	0	0
9	*Ae. aegypti*	CAM	7.6	31.8 (38)	58.3	8.3
		PR	7.6	59.4 (32)	68.4	5.3
14	*Ae. aegypti*	CAM	7.6	22.6 (31)	71.4	14.3
		PR	7.6	45(40)	77.8	16.7
	Ae. albopictus	CAM	7.6	81.5 (27)	81.8	9.1
		PR	7.6	93.3 (30)	67.9	0
	Ae. caspius	PR	8.7	40 (25)	0	0
21	*Ae. aegypti*	CAM	7.6	35.7 (28)	100	40
		PR	7.6	56.3 (32)	88.9	38.9
	Ae. albopictus	CAM	7.6	94.4 (18)	82.4	23.6
		PR	7.6	96.2 (26)	96	36
	Ae. caspius	PR	7.6	18.5 (27)	0	0

Table 2. Results of GLMs analyzing the variation in infection, dissemination and transmission rates in relation to mosquito species, ZIKV strains and days post infection (dpi). The interactions between different variables are indicated with *. Significant effects are highlighted in bold.

	Infection rate			Dissemination rate			Transmission rate		
Variables	$\chi2$	d.f.	*p*	$\chi2$	d.f.	*p*	$\chi2$	d.f.	*p*
Mosquito species	110.95	1	**<0.001**	2.08	1	0.15	2.37	1	0.12
ZIKV strain	10.43	1	**0.001**	1.28	1	0.26	4.91	1	**0.03**
Dpi	0.15	1	0.70	39.61	1	**<0.001**	26.77	1	**<0.001**
ZIKV strain*dpi	1.17	1	0.28	1.34	1	0.25	6.70	1	**0.01**
Mosquito species*ZIKV strain	0.01	1	0.90	0.76	1	0.39	0.01	1	0.94

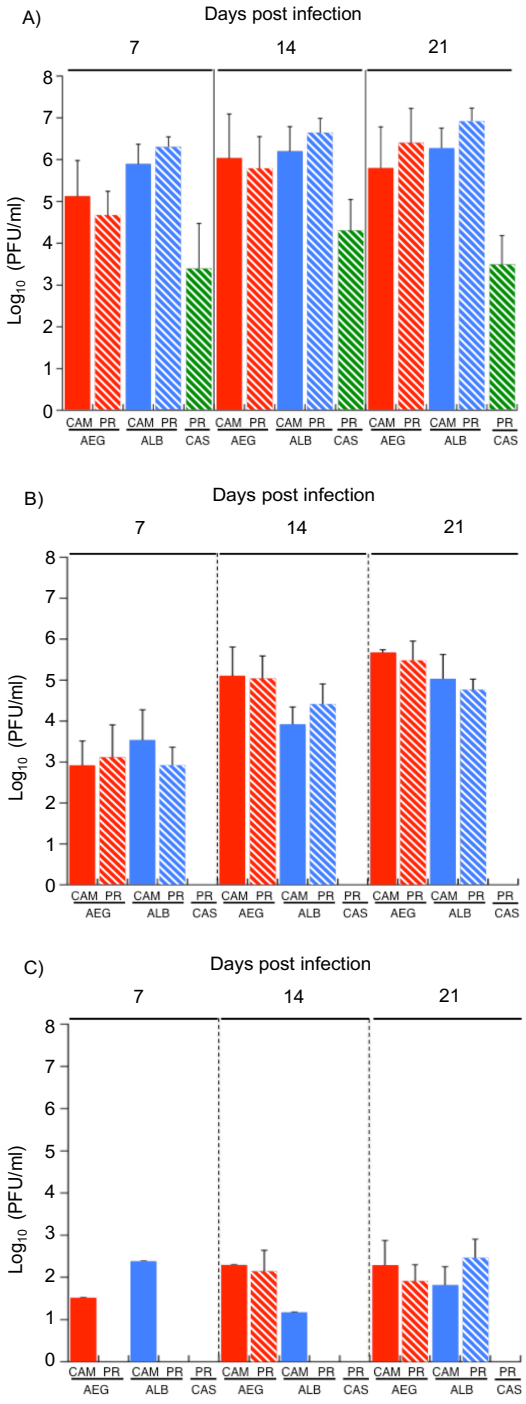

Fig. 1. Mean ± SE ZIKV CAM and ZIKV PR titers (Log_{10} (PFU/ml) in the body (A), legs (B) and saliva (C) of *Aedes caspius* (CAS), *Aedes albopictus* (ALB) and *Aedes aegypti* (AEG) at 7, 14 and 21 days post infection.

Discussion

Our results demonstrate that, although ZIKV can infect *Ae. caspius*, this virus is unable to disseminate through the mosquito's midgut. Consequently, *Ae. caspius* is unlikely to transmit ZIKV when naturally infected, and only a small fraction of these mosquitoes could transmit the virus after intrathoracic inoculation. This suggests that *Ae. caspius* has a midgut escape barrier, which prevents that the virus be able to disseminate throughout the body of the mosquito and achieve the salivary glands (Hardy *et al.* 1983). Spanish *Ae. albopictus*, however, had similar vector competence to *Ae. aegypti* for the transmission of two ZIKV strains currently circulating in America and Asia.

We found that ZIKV CAM had higher transmission rates and was detected in saliva before ZIKV PR, a pattern that could be explained by genetic differences between both ZIKV strains (Ciota *et al.* 2017). Although *Ae. albopictus* from France showed a transmission rate of only 4.17% (Jupille *et al.* 2016), populations from Italy and Germany can transmit ZIKV with similar rates to that reported in this study at 14 and 21dpi (Di Luca *et al.* 2016; Heitmann *et al.* 2017). Nonetheless, our results showed that the Spanish population of *Ae. albopictus* could transmit ZIKV from 7dpi, four days earlier than previously reported (Di Luca *et al.* 2016). It is known that vector competence may vary between virus strains and mosquito populations (Ciota *et al.* 2017), likely explaining discrepancies found between studies.

We found strong evidence for vertical transmission of ZIKV in *Ae. albopictus*, with a ratio of 1:14, which is substantially higher than what previously found for ZIKV in *Ae. albopictus* from New York (Ciota *et al.* 2017) as well as for other flaviviruses (Thangamani *et al.* 2016). However, vertical transmission was not found in *Ae. albopictus* from Italy (Di Luca *et al.* 2016). Despite these differences, results found in different mosquito populations strongly support the ability of ZIKV to be transmitted vertically, which represents a potential mechanism for virus maintenance under natural conditions in seasonal environments.

In conclusion, our results confirm that the invasive populations of *Ae. albopictus* increase the risk of ZIKV transmission in Europe. This is especially relevant in light of the rapid spread of *Ae. albopictus* in the Mediterranean basin, including Spain, and the high

number of imported ZIKV cases in the area. In contrast, the risk of ZIKV transmission by *Ae. caspius* may be considered extremely low.

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Chapter 3

Do mosquitoes transmit the avian malaria-like parasite *Haemoproteus*? An experimental test of vector competence using mosquito saliva

Rafael Gutiérrez-López, Josué Martínez-de la Puente, Laura Gangoso, Jiayue Yan, Ramón Soriguer, Jordi Figuerola. (*Parasites & vectors, 2016; 9:609*)

Abstract

The life-cycle of many vector-borne pathogens includes an asexual replication phase in the vertebrate host and sexual reproduction in the insect vector. However, as only a small array of parasites can successfully develop infective phases inside an insect, few insect species are competent vectors for these pathogens. Molecular approaches have identified the potential insect vectors of blood parasites under natural conditions. However, the effectiveness of this methodology for verifying mosquito competence in the transmission of avian malaria parasites and related Haemosporidians is still under debate. This is mainly because positive amplifications of parasite DNA in mosquitoes can be obtained not only from sporozoites, the infective phase of the malaria parasites that migrate to salivary glands, but also from different non-infective parasite forms in the body of the vector. Here, we assessed the vectorial capacity of the common mosquito *Culex pipiens* in the transmission of two parasite genera. A total of 1,560 mosquitoes were allowed to feed on five house sparrows *Passer domesticus* naturally infected by *Haemoproteus* or co-infected by *Haemoproteus*/*Plasmodium*. A saliva sample of the mosquitoes that survived after 13 days-post-exposure was taken to determine the presence of parasite DNA by PCR. Overall, 31.2% mosquito's head-thorax and 5.8% saliva samples analysed showed positive amplifications for avian malaria parasites. In contrast to *Haemoproteus* DNA, which was not found in either the body parts or the saliva, *Plasmodium* DNA was detected in both the head-thorax and the saliva of mosquitoes. Parasites isolated from mosquitoes feeding on the same bird corresponded to the same *Plasmodium* lineage. Our experiment provides good evidence for the competence of *Cx. pipiens* in the transmission of *Plasmodium* but not of *Haemoproteus*. Molecular analyses of saliva are an effective method for testing the vector competence of mosquitoes and other insects in the transmission of vector-borne pathogens.

Introduction

The avian malaria parasite *Plasmodium* and the malaria like parasites of the genus *Haemoproteus* are pathogens that infect birds worldwide and cause infectious diseases that affect birds' fitness (Merino *et al.* 2000; Marzal *et al,* 2005). These parasites reproduce asexually in birds but are obliged to complete their sexual and sporogonic phases in their insect vectors before being successfully transmitted to a new vertebrate host. Mosquitoes (Diptera: Culicidae), especially those of the genus *Culex*, are the main vectors of avian *Plasmodium*; biting midges *Culicoides* (Diptera: Ceratopogonidae) and louse flies (Diptera: Hippoboscidae), on the other hand, transmit *Haemoproteus* (subgenera *Parahaemoproteus* and *Haemoproteus*) parasites, respectively (Atkinson & Van Riper, 1991; Valkiūnas, 2005). In mosquitoes, after the development of the ookinetes, parasites penetrate insects' mid-gut walls and produce oocysts. These oocysts then divide to produce the sporozoites, the infective form of the malaria parasites, which migrate to the salivary glands of the mosquitoes. Sporozoites are thus transmitted by mosquito bites into the bloodstream of a new host (Valkiūnas, 2005). Since the seminal paper by Bensch *et al.* (2000), a number of different molecular approaches have been developed to study interactions between parasites and birds (Bensch *et al.* 2000; Martínez *et al.* 2009). These molecular methods are also a valuable tool for identifying the potential insect vectors of blood parasites under natural conditions (Ishtiaq *et al.* 2008; Levin *et al.* 2012). However, an intense debate exists regarding the reliability of molecular approaches in the study of vector competence (Valkiūnas, 2014; Seblova *et al.* 2014). This controversy arises from the fact that positive amplification of parasite DNA can be obtained from insects due to the presence of non-infective forms of the parasite, which are unable to complete their multiplicative cycle. For instance, *Haemoproteus* DNA has been isolated from both *Culicoides* (Martínez-de la Puente *et al.* 2011; Ferraguti *et al.* 2013 a) and several mosquito species, including *Culex pipiens*, which have completely digested blood meals (Santiago-Alarcón *et al.* 2012; Ferraguti *et al.* 2013 b; Synek *et al.* 2013; Zélé *et al.* 2014). All this evidence suggests that mosquitoes (and not only *Culicoides*) could be involved in the transmission of this parasite genus. Therefore, further studies are still required to determine the degree to which mosquitoes are competent in the transmission of *Haemoproteus* parasites. We conducted an experimental study to determine, to our knowledge for the first

time, the competence of *Cx. pipiens* mosquitoes in the transmission of avian malarialike parasites of the genus *Haemoproteus*. *Culex pipiens* is a widely distributed mosquito species involved in the transmission of a number of vector-borne pathogens (Farajollahi *et al.* 2011). It is believed to be one of the main vectors of avian malaria parasites, and over 50 different genetic lineages have been detected in this mosquito species using molecular methods (Santiago-Alarcón *et al.* 2012; Bensch *et al.* 2009). To assess vector competence, mosquitoes were allowed to feed on wild birds naturally infected by *Haemoproteus* and birds co-infected by *Haemoproteus* and *Plasmodium* (individuals suffering co-infections are commonly found in the wild) (Marzal *et al.* 2008; Merino *et al.* 2008; Del Cerro *et al.* 2010). After allowing the parasite to develop in the mosquito, we used molecular tools (PCR) to detect the presence of parasite DNA in the head-thorax (where the salivary glands are located) and saliva of mosquitoes. The detection of pathogens in mosquito saliva is frequently used in studies of the vector competence of pathogens such as West Nile virus (Goddard *et al.* 2002) and Chikungunya virus (Dubrulle *et al.* 2009) but, to the best of our knowledge, has never previously been employed to determine the vector competence of mosquitoes for avian malaria and malaria-like parasites.

Material and methods

Mosquito collection and rearing

Culex pipiens larvae were collected in La Cañada de los Pájaros, a natural reserve near Seville, Spain (6°14′W, 36°57′N). This area lies beyond the main wetlands of the Doñana National Park and consists of a freshwater lake (c.5 ha) surrounded by paddy fields. Larvae were transferred to the laboratory and kept in plastic trays with fresh water and fed ad libitum (Mikrozell 20 ml/22 g; Dohse Aquaristik GmbH & Co. KG, D-53501, Gelsdorf, Germany). Larvae and adult mosquitoes were maintained at constant conditions, 28 °C, 65–70% relative humidity (RH) and 12:12 light: dark cycle. After metamorphosis, adult mosquitoes were immediately placed in insect cages (BugDorm-43030F, 32.5 × 32.5 × 32.5 cm) and fed ad libitum with 1% sugar solution. Five to seven days later, adults were anesthetised with ether (Lipnick, 1991) and observed under a stereomicroscope (Nikon SMZ645) to determine their sex and confirm the species, following Schaffner *et al.* (2001) and Becker *et al.* (2010). The sugar solution was replaced with water 24 h prior to each

experiment (see below) and completely removed from cages 12 h before experiments began. The experiments were conducted using 13–22-day-old female *Cx. pipiens.*

Bird trapping and sampling

Five juvenile (yearlings) house sparrows *Passer domesticus* were captured using mist nets on 15 July 2014 in Huelva province and subsequently ringed with numbered metal rings. To determine their haemosporidian infection status, a blood sample (0.2 ml) was taken from the jugular vein of each bird using sterile syringes and was then immediately transferred to non-heparinized Eppendorf tubes. Birds were transported to the Unit of Animal Experimentation at the Estación Biológica de Doñana (EBD-CSIC) and kept indoors in birdcages (58.5 × 25 × 36 cm) in a vector-free room under controlled conditions (23 ± 1 °C, 40–50% RH and 12:12 light: dark cycle). Birds were fed ad libitum with a standard mixed diet for seed-eating and insectivorous birds (KIKI; GZM S.L., Alicante, Spain). Three days after the last exposure to mosquitoes, birds were blood sampled again (0.2 ml; final blood samples) in the same way as above to detect any infections by blood parasites that could have not developed when initially sampled. Samples were not taken either immediately before or during the mosquito exposure period due to the stress caused by mosquito bites. Immediately after sampling, a drop of blood was smeared, air-dried, fixed in absolute methanol and stained with Giemsa for 45 min (Gering *et al.* 2004). A total of 4,000–10,000 erythrocytes from each blood smear were scanned at high magnification (×1000) and the intensity of infection by *Haemoproteus*/*Plasmodium* parasites was estimated as the percentage of parasite cells per 100 erythrocytes. At the end of the experiment, birds were released at the capture site 23 days after being captured.

Experimental procedure

Eleven days after capture, each bird was placed in a birdcage (38.5 × 25.5 × 26 cm) inside an insect tent (BugDorm-2120, 60 × 60 × 60 cm). Over four non-consecutive nights, each bird was introduced into an independent tent and exposed to 50 (first night), 57 (second night), 105 (third night) and 100 (fourth night) unfed *Cx. pipiens* females, summarizing a total of 312 mosquitoes per bird. The number of mosquitoes used each night varied according to the availability of unfed 13–22 days old mosquitoes. Birds were

exposed to mosquito bites overnight (from 8:00 pm to 8:00 am). After exposure, mosquitoes with a recent blood meal in the abdomen were immediately separated and placed in unzipped insect cages (BugDorm-43030F 32.5 × 32.5 × 32.5 cm) and maintained under standard conditions (28 °C, 65–70% RH and 12:12 light: dark cycle). These mosquitoes had ad libitum access to 1% sugar solution during the following 13 days to allow parasite development.

Sampling of mosquito saliva

Those mosquitoes that survived until 13 days post-exposure (dpe) were anesthetised with ether (Lipnick, 1991). Mosquitoes' legs and wings were removed with sterile forceps. The mosquito proboscis was introduced into a 1µl disposable capillary (Einmal-Kapillarpipetten, Hirschmann® Laborgeäte, Germany) filled with 1µl of fetal bovine serum (Phillips *et al.* 2010). Then, 1µl of 2% pilocarpine (Novartis 2012, Alcon Cusí S.A. Barcelona, Spain) was applied to the mosquito thorax to stimulate salivation (Boorman 1987). After 45 min, the medium containing the saliva was placed in 1.5ml Eppendorf tubes with 10µl of MQ water and stored at −80 °C. Mosquitoes were kept in individual tubes at -80 °C until further molecular analysis. The head-thorax of eight mosquitoes and two saliva samples were not analysed due to logistical problems.

Molecular detection and identification of blood parasites

DNA was isolated from birds' blood samples (both the initial and final samples) and from the head-thorax of mosquitoes using a semi-automatic procedure (MAXWELL® 16 LEV Blood DNA Kit) (Gutiérrez-López *et al.* 2015). The Qiagen DNeasy® Kit Tissue and Blood (Qiagen, Hilden, Germany) was used to isolate the DNA from saliva samples. A 478 bp fragment (excluding primers) of the mitochondrial cytochrome b gene of *Haemoproteus*/*Plasmodium* parasites was amplified following Hellgren *et al.* (2004). This procedure is based on a first PCR using primers HaemNFI (5′-CAT ATA TTA AGA GAA ITA TGG AG-3′) and HaemNR3 (5′-ATA GAA AGA TAA GAA ATA CCA TTC-3′), followed by a nested PCR using primers HaemF (5′-ATG GTG CTT TCG ATA TAT GCA TG-3′) and HaemR2 (5′-GCA TTA TCT GGA TGT GAT AAT GGT-3′). This procedure is able to detect parasite DNA in infections equivalent to less than one gametocyte per

10,000 erythrocytes in blood smears (Hellgren *et al.* 2004). The presence of amplicons was verified in 1.8% agarose gels. Positive amplifications were sequenced in both directions using the BigDye technology (Applied Biosystems) or with the Macrogen sequencing service (Macrogen Inc., Amsterdam, The Netherlands). Sequences were edited using the software Sequencher™ v 4.9 (Gene Codes Corp. © 1991–2009, Ann Arbor, MI 48108, USA) and assigned to parasite lineages/morphospecies after comparison with the GenBank (National Center for Biotechnology Information) and Malavi (Bensch *et al.* 2009) databases.

Results

The five birds included in the study showed positive amplifications of blood parasites and there was no difference between initial and final samples. The parasite sequences isolated from all five birds had a 100% overlap with lineage *Haemoproteus* PADOM05 (corresponding to *H. passeris*). No evidence of double peaks in the chromatograms was found. The examination of blood smears revealed the presence of both *Haemoproteus* and *Plasmodium* parasites in two birds (house sparrows 4 and 5), only *Haemoproteus* in two other birds (house sparrows 2 and 3), and a total absence of parasites in one bird (house sparrow 1) (Fig. 1, Table 1).

Overall, 174 of 1560 (11.2%) mosquitoes used in this study fed on birds' blood, 149 of them survived until 13 dpe. A total of 141 head-thorax and 139 saliva samples were molecularly analysed, of which 44 and 8 samples, respectively, were positive to parasite DNA (Table 1). All the saliva samples showing positive amplifications corresponded to mosquitoes with head-thorax that were also positive for parasite DNA.

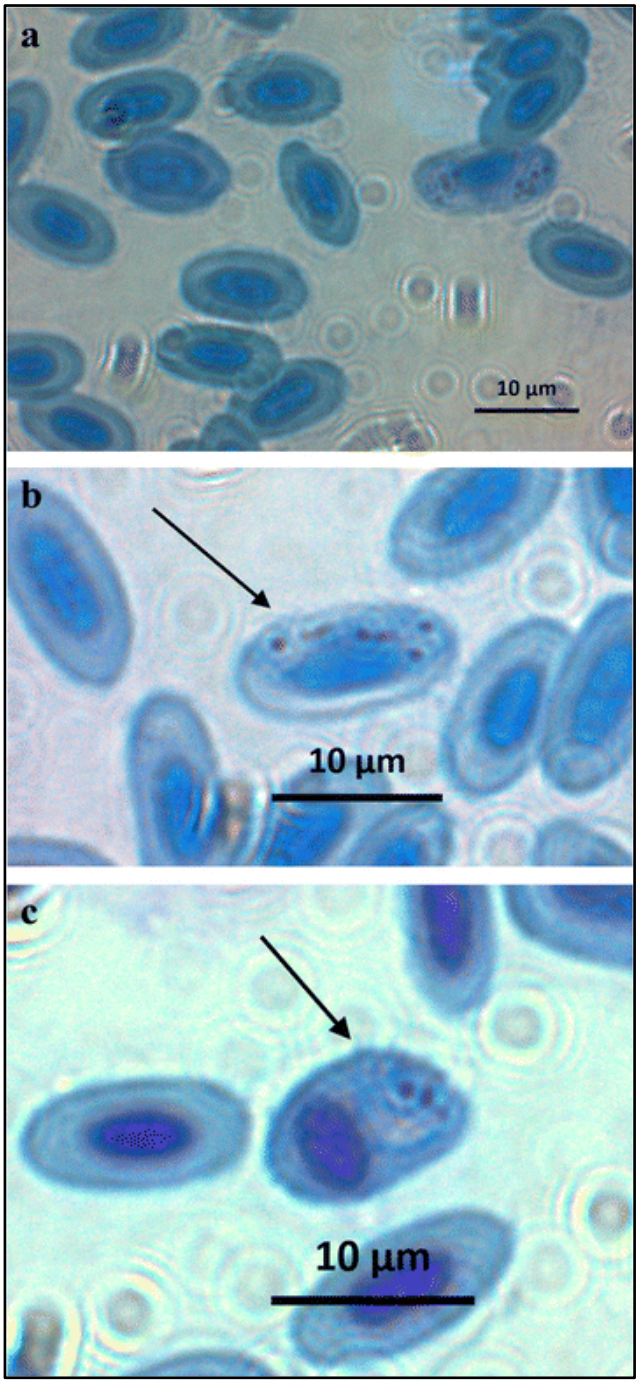

Fig. 1. Blood parasites found in house sparrows (a) with details of *Haemoproteus passeris* (lineage padom05) (b) and *Plasmodium sp.* lineage PADOM01 (c). *Arrows* indicate the parasite cell

The parasite lineages isolated from the head-thorax and saliva of the mosquitoes that fed on the two co-infected birds, as revealed by the blood smears, corresponded to *Plasmodium* lineages. These lineages were identified as SGS1 (also named Rinshi-1, corresponding to *Plasmodium relictum*) and PADOM01. We were unable to detect *Plasmodium* in the blood smear of one bird (identified as house sparrow 3, Table 1), probably due to a very low-intensity of infection but did manage to isolate the *P. relictum* lineage GRW11 (= Rinshi-7) in the head-thorax of one of the 36 mosquitoes that fed on this bird (Table 1). Parasites isolated from mosquitoes feeding on the same individual corresponded to the same *Plasmodium* lineage. *Haemoproteus* was not found in either the head-thorax or in the saliva of any of the mosquitoes analysed.

	Infection status (PCR)	Intensity of infection and morphological identification of parasites (blood smear)	Engorged mosquitoes	Alive mosquitoes after 13 days	Head-thorax positive/analysed	Saliva positive/analysed
House sparrow 1	*Haemoproteus*	*Haemoproteus* (0%)	9 (2.9%)	9 (100%)	0/9	0/9
House sparrow 2	*Haemoproteus*	*Haemoproteus* (0.4%)	39 (12.5%)	35 (89.7%)	0/34	0/34
House sparrow 3	*Haemoproteus*	*Haemoproteus* (0.2%)	42 (13.5%)	36 (85.7%)	1/36	0/36
House sparrow 4	*Haemoproteus*	*Haemoproteus* (0.5%) / *Plasmodium* (0.2%)	39 (12.5%)	33 (84.6%)	23/26	7/26
House sparrow 5	*Haemoproteus*	*Haemoproteus* (1.3%) / *Plasmodium* (0.3%)	45 (14.4%)	36 (80%)	20/36	1/34

Table 1. Infection status of birds included in this study and number of engorged and analyzed *Culex pipiens* mosquitoes

Discussion

Studies of host-parasite co-evolution in the context of avian malaria mainly focus on the interactions between parasites and their vertebrate hosts (Charleston & Perkins, 2003; Pérez-Tris *et al.* 2008; Martinsen *et al.* 2008) but tend to ignore the role of invertebrate vectors. The development of avian blood parasites in mosquitoes is the outcome of a complex evolutionary 'arms race' too, in which the probability of encounter with mosquitoes and their compatibility are important obstacles for successful infection and the proper development of the parasites (Gilbert & Webb 2007; Medeiros *et al.* 2013).

Although *Cx. pipiens* females frequently feed on mammals, birds are their main blood-feeding source (Farajollahi *et al.* 2011; Muñoz *et al.* 2012; Martínez-de la Puente *et al.* 2015), a preference that may increase their contact rate with *Haemoproteus*. Nevertheless, our results suggest that mosquitoes actually may represent an obstacle to the successful development of the life-cycle of species in this parasite genus (Medeiros *et al.* 2013). Here, we provide evidence of the effectiveness of mosquito saliva as a novel way of testing the vectorial competence of mosquitoes in the transmission of avian malaria and malaria-like parasites. This method has been commonly used in studies of the vector competence of mosquitoes in the transmission of a number of viruses that are of public health concern (Aitken, 1977; Colton *et al.* 2005; Vazeille *et al.* 2010; Vogels *et al.* 2016) as well as to detect proteins of *Plasmodium bergehi* sporozoites in the saliva of *Anopheles stephensi* (Golenda *et al.* 1992). However, to our knowledge, this approach has never been used in studies of mosquito-avian malaria interactions. Despite being time-consuming (it is possible to obtain the saliva of about 15 mosquitoes/h), this method is an excellent complementary procedure to the frequently used salivary gland dissection employed in studies on vector competence. By using this approach, it is possible to obtain parasite sporozoites while reducing/removing the presence of tissues derived from the salivary glands present in the sample. This could be of special relevance in studies on *Plasmodium* genotyping where the quantity of parasite DNA in relation to host DNA is an important limitation (Schall & Vardo, 2007). Moreover, mosquito saliva could be used in transcriptomic studies of the infective forms of avian malaria parasites and/or to study the parasite load inoculated by mosquitoes (Kappe *et al.* 2001).

The lineages SGS1 (*P. relictum*) and PADOM01 were amplified in the saliva of mosquitoes at 13 dpe. However, a high percentage of mosquitoes with positive DNA amplifications in the head-thorax (81.8%) did not show positive *Plasmodium* DNA amplifications in saliva at 13 dpe. A recent study found that 13.3% of infected *Cx. pipiens* had *Plasmodium* sporozoites in their salivary glands (Palinauskas *et al.* 2016), indicating that these parasites develop sporozoites in only a small percentage of infected mosquitoes. The absence of sporozoites in salivary glands could be explained by the fact that the parasite does not have enough time to complete its development until this phase. Thus, extracting saliva after 13 dpe could have increased the number of positive amplifications in our

samples. However, some studies have found *Plasmodium* sporozoites in the salivary glands of mosquitoes from just 7 dpe (Valkiūnas, 2005; Christensen *et al.* 1983), although Kazlauskienė *et al.* (2013) were unable to isolate sporozoites until 14 dpe in salivary glands (yet mosquitoes at 13 dpe were not analysed). The differences found between studies could be due to the use of different mosquito species, a differential mosquito microbiota, parasite strains, or environmental temperatures, which may greatly affect the ability of parasites to complete sporogony (Valkiūnas, 2005; Dong *et al.* 2009; Murdock *et al.* 2012). Unlike *Plasmodium*, the possibility that *Haemoproteus* had not have enough time to develop sporozoites is poorly supported. Previous studies using direct observational (microscope) and molecular (PCR) techniques found intermediate stages (i.e. ookinetes and oocysts) of *Haemoproteus* parasites in the head, thorax and/or abdomen of *Ochlerotatus cantans* mosquitoes from 4–6 dpe onwards, but presence of sporozoites was not recorded (Valkiūnas *et al.* 2013; (Valkiūnas *et al.* 2014). By contrast, we found no evidence of *Haemoproteus* DNA in the head-thorax of the mosquitoes analysed. In addition, in their known *Culicoides* vectors, *Haemoproteus* sporozoites are also present in salivary glands at 5 dpe (Valkiūnas *et al.* 2002). Therefore, our results support the inability of *Haemoproteus* lineage PADOM05 to complete its lifecycle in *Cx. pipiens*.

Molecular approaches allowing the identification of the parasite lineages harboured by insect vectors provide valuable information on the potential transmission networks of avian pathogens (Martínez-de la Puente *et al.* 2011; Santiago-Alarcón *et al.* 2012; Synek *et al.* 2013; Zélé *et al.* 2014). Such tools enable a huge number of individuals (e.g. thousands of mosquitoes) to be handled, which is often necessary for detecting positive amplifications due to the low infection prevalence that is typical in mosquitoes trapped in the wild (Ferraguti *et al.* 2013 b, Glaizot *et al.* 2012; Larcombe & Gauthier-Clerc, 2015). However, results from these studies should be interpreted with caution when attempting to identify the true vectors of avian pathogens, this is especially true when pathogen DNA is isolated from an unexpected vector, and highlights the necessity to conduct further experimental studies of vectorial competence (Valkiūnas, 2014). Although different approaches including cloning and the development of specific primers have been employed to identify parasite lineages in co-infected birds (Martínez *et al.* 2009; Perez-Tris & Bensch, 2005; Bernotienė *et al.* 2016), our results show the importance of

combining the molecular detection of blood parasites with the analysis of blood smears when aimed at identifying potential co-infections in birds (Valkiūnas *et al.* 2006).

Conclusions

The results from this study suggest that *Cx. pipiens* is unable to transmit *Haemoproteus* parasites. This study also highlights the value of targeting mosquito saliva as a means of assessing the competence of potential mosquito vectors in the transmission of avian *Plasmodium* lineages.

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Chapter 4

Plasmodium transmission risk differs between mosquito species and parasite lineages: effects on vector survival and parasite development

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Abstract

Mosquito competence for the transmission of malaria parasites may be strongly affected by the particular *Plasmodium*-mosquito assemblages and the parasite load in the vertebrate host. Here, we assessed the competence of two mosquito species, i.e *Culex pipiens* and *Ochlerotatus caspius*, with different feeding patterns, for the transmission of four avian *Plasmodium* lineages infecting wild house sparrows. The lineages identified corresponded to the lineages SGS1 and GRW11 belonging to *Plasmodium relictum* (clade A in this study) and the lineage COLL1 and PADOM01, named clade B in this study. In addition, we assessed the effects of parasite identity (i.e. clade identity) and parasite load on the transmission rate of avian *Plasmodium* by mosquitoes. We also analyzed the impact of *Plasmodium* infection on mosquito survival as well as parasite development on the *Plasmodium* transmission risk. We found that *Cx. pipiens* was able to transmit the four *Plasmodium* lineages, but *Oc. caspius* was unable to transmit any of these parasite lineages. However, the parasite transmission rate and cost on *Cx. pipiens* survival differed between the two *Plasmodium* clades. Mosquitoes fed on birds infected by parasites of the clade A showed a lower survival and transmission rate than those fed on birds infected by parasites of the clade B, while non-significant associations were found with bird parasite load. Our results confirm the existence of inter- and intra-specific differences in the ability of *Plasmodium* lineages to develop in mosquito species. The differences in the transmission rate and virulence between parasites of clades A and B led to differences in the transmission risk, with higher rates found for *Plasmodium* lineages of clade B.

Introduction

Parasites of the genus *Plasmodium*, the causative agents of malaria, are vector-borne Haemosporidians that largely affect humans and wildlife (Sachs & Malaney, 2002; Valkiūnas, 2005). *Plasmodium* parasites are transmitted by mosquitoes, in which they develop the sexual reproductive phase (Valkiūnas, 2005). Consequently, mosquito-*Plasmodium* interactions play an important role in the dynamics of parasite transmission (Kimura *et al*. 2010).

Avian *Plasmodium* shows a wide range of competent hosts belonging to different bird families and orders (Fallon *et al*. 2005; Pérez-Tris *et al*. 2007; Hellgren *et al*. 2009). Mosquitoes of the genus *Culex* are the main vectors of avian malaria parasites, although other genera such as *Aedes* or *Anopheles* could be involved in their transmission (Santiago-Alarcón *et al*. 2012). Genetically related parasite lineages/species have been detected in different mosquito genera, suggesting a generalist relationship between *Plasmodium* and mosquitoes (Kimura *et al*. 2010; Ferraguti *et al*. 2013; Schoener *et al*. 2017). Most information regarding the vector species involved in avian *Plasmodium* transmission is based on the molecular identification of parasite DNA from wild mosquitoes, without a quantitative evaluation of vector/parasite associations (Kimura *et al*. 2010; Ferraguti *et al*. 2013; Schoener *et al*. 2017). However, molecular detection of parasite DNA from insects does not imply these are competent vectors (Martínez-de la Puente *et al*. 2012; Santiago-Alarcón *et al*. 2012). In addition, interspecific differences in competence for the transmission of avian *Plasmodium* could be underestimated, as parasite DNA may also be isolated from non-competent mosquito species (Beerntsen *et al*. 2000; Ishtiaq *et al*. 2008). Thus, it becomes crucial to quantitatively evaluate the actual 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 as to allow parasites to complete their life cycle (between 8-13 days; Valkiūnas, 2005; LaPointe *et al*. 2010). The development of *Plasmodium* in the mosquito may be affected by a great variety of abiotic environmental factors, such as temperature and humidity (Paaijmans *et al*. 2010; Lefévre *et al* 2013). In addition, biotic factors including the parasite load in the vertebrate host (Cornet *et al*. 2014) and the particular parasite-vector

assemblage may further determine the success of development of parasites in mosquitoes. For example, mosquitoes feeding on birds with high parasite loads develop a higher density of ookinetes (a previous 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 & Villa 2018). However, most of these studies have focussed on the interaction between *Culex pipiens* mosquitoes and *Plasmodium relictum* (lineage SGS1). Therefore, studies considering potential differences in virulence (i.e. the cost of the pathogen infections on their host) between parasite species/lineages on different vector mosquito species are necessary (see Lachish *et al*. 2011).

Here, we experimentally assessed the competence of two mosquito species *Culex pipiens* and *Ochlerotatus caspius* for the transmission of different avian *Plasmodium* lineages. Both mosquito species are common in Southern Spain where they show different feeding patterns. While *Culex pipiens* feed mainly on birds (Martínez-de la Puente *et al*. 2015), *Oc. caspius* prefers to bite mammals, although birds may represent up to 19% of their diet (Balenghien *et al*. 2006; Muñoz *et al*. 2012). 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 is well demonstrated (Gutiérrez-López *et al*. 2016; Palinauskas *et al*. 2016; Kazlauskienė *et al*. 2013). In this study, mosquitoes were allowed to feed on *Plasmodium* infected birds to assess the effects of avian parasite load and parasite identity on the probability of mosquito infection and parasite transmission. We also analyzed 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) (Ross, 1911; Macdonald, 1955).

Materials and methods

Mosquito collection and rearing

Wild larvae of *Cx. pipiens* and *Oc. 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), respectively. Larvae were grown in plastic trays with fresh or brackish water, respectively, and fed *ad libitum* with Mikrozell 20ml/22g (Dohse Aquaristik GmbH & Co.101 KG, D-53501, Gelsdorf, Germany). Larvae and emerged imagos were maintained at standard conditions (28ºC ± 1, 65–70% relative humidity (RH) and 12:12 light:dark photoperiod cycle). Adult mosquitoes were anesthetized with ether and subsequently sexed and identified to species level based on morphology (Schaffner *et al*. 2001). Female mosquitoes were 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-weeks-old female mosquitoes were deprived from sugar solution. Laboratory maintained colonies of mosquitoes were not used to minimize the effects of artificial selection of mosquitoes with particular biting preferences (Franks *et al*. 2001; Lagisz *et al*. 2011).

Bird sampling and experimental procedure

Fifty-five 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 the Huelva Province using mist nets. Birds were individually ringed and weighed. These birds plus sixteen additional house sparrows from another study (see below) were kept indoor in birdcages (58.5 × 25 × 36 cm) in a vector-free room under controlled conditions (23 ± 1 °C, 40–50% RH and 12:12 light: dark cycle) in the Unit of Animal Experimentation at Estación Biológica de Doñana (EBD-CSIC). The 16 birds had been injected with saline solution in the context of a different study addressing the effect of parasite load on host selection by mosquitoes (Yan *et al*. 2017). Since no effects of this treatment are expected to influence the biting patterns of mosquitoes, and in accordance with animal ethical and welfare policies, these birds were used in this experiment before being released. A blood sample was obtained from the jugular vein of each bird using sterile syringes (0.2 ml). A drop of blood was smeared, air-dried, fixed with absolute methanol

and stained with Giemsa for 45 min (Gering & Atkinson, 2004). The rest of the blood sample was transferred to non-heparinized Eppendorf tubes to perform molecular detection of parasites (see below). A total of 4000–10000 erythrocytes from each smear were scanned at high magnification (x1000) and *Plasmodium* parasite load was estimated as the percentage of parasite cells per 100 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).

Individual birds were enclosed for 30 minutes in an insect cage (BugDorm-43030F, 32.5×32.5×32.5 cm) containing either *Cx. pipiens* or *Oc. caspius* mosquito females. Birds were immobilized to prevent defensive behaviours against mosquitoes using a cylinder made with 1×1 cm mesh, allowing mosquitoes to bite through. At the end of each trial, birds were released without any apparent sign of damage. After trials, mosquitoes with a recent blood meal in the abdomen were immediately separated and placed in unzipped insect cages (BugDorm-43030F 32.5 x 32.5 x 32.5 cm) and maintained under the same conditions detailed above. Mosquito survival was monitored every 12h until 13 days post-exposure. At the end of this period, the saliva of surviving mosquitoes was obtained as in Gutiérrez-López *et al*. (2016). Briefly, the mosquito proboscis was introduced into a 1µl disposable capillary (Einmal-Kapillarpipetten, Hirschmann® Laborgeäte, Germany) with 1µl of foetal bovine serum. One µl of 2% pilocarpine (Novartis 2012, Alcon Cusí S.A. Barcelona, Spain) was applied to the mosquito thorax to stimulate salivation. After 45 min, the medium containing the saliva was placed in 1.5 ml Eppendorf tubes with 10µl of MQ water. Samples were kept at -80 ºC until further molecular analyses.

Molecular analyses

Genomic DNA was isolated from bird blood samples using the MAXWELL ® 16 LEV Blood DNA Kit (Gutiérrez-López *et al*. 2015). The head-thorax of each mosquito, containing the salivary glands, was separated from the abdomen, legs and wings in a sterile Petri dish. The genomic DNA from the head-thorax of mosquitoes was extracted using the same procedure described above. The Qiagen DNeasy® Kit Tissue and Blood (Qiagen, Hilden, Germany) was used to isolate the DNA from saliva samples. A 478 bp fragment

(excluding primers) of the mitochondrial cytochrome b gene of *Plasmodium/ Haemoproteus* parasites was amplified following Hellgren *et al*. (2004). The presence of amplicons was verified in 1.8% agarose gels. Positive amplifications were sequenced in both directions using the BigDye technology (Applied Biosystems) or with the Macrogen sequencing service (Macrogen Inc., Amsterdam, The Netherlands). Sequences were edited using the software Sequencher™ v4.9 (Gene Codes Corp. © 1991–2009, Ann Arbor, MI 48108, USA) and assigned to parasite lineages/morphospecies after comparison with the GenBank (National Center for Biotechnology Information) and MalAvi databases (Bensch *et al*. 2009). Birds with coinfections were not included in this study to avoid potential confounding effects of multiple infections on parasite development and mosquito survival (see Lover *et al*. 2015).

Ethics statements

All experiments involving birds adhered to the guidelines included in the Spanish Legislative Decree "Real Decreto 53/2013 de 1 de Febrero" on protection of animals used for experimentation and other scientific purposes, with the guidelines established by the European Community Council Directive n° 2010/63/UE on Laboratory Animal Protection. Regional Authorities and the CSIC Ethics Committee approved this project (ref. CEBA-EBD-12-40). Mosquito sampling was done with all the necessary permits from landowners and regional Department of the Environment (Consejería de Medio Ambiente, Junta de Andalucía).

Statistical analyses

The four *Plasmodium* lineages found in this study were grouped into two main clades (clades A and B, see results) and uncorrected p-distances between lineages/clades were compared using MEGA7 Software (Kumar *et al*. 2016). We fitted two similar Generalized Mixed Linear Models (GLMMs) with binomial error and logit link function to assess the effects of *Plasmodium* clade identity (fixed factor) and the bird parasite load (covariable) on the status of *Plasmodium* infection (infected/non-infected) in the head-thorax or saliva, respectively. In both models, bird identity was included as a random term. The bird parasite load was log-transformed to attain normality. We fitted a Cox mixed-

effect model by maximum likelihood to assess the effect of parasite infection status (infected/un-infected birds) and bird parasite load on mosquito survival (measured as the number of surviving mosquitoes per 12-hours-periods), while controlling for the potential effect of mosquito age (2 or 3 weeks old). We also conducted other independent Cox mixed-effect model using parasite identity (clade A, clade B and controls, see below) instead of parasite infection status as the dependent variable. We restricted these analyses to *Cx. pipiens* mosquitoes as parasite development was only observed in this species (see results). In these analyses, we included data from mosquitoes fed on 10 un-infected house sparrows as controls. Statistical analyses were performed in R software 3.2.5 (R Core Development Team, 2016) with the package *lme4* (Bates *et al*. 2015).

Modelling Plasmodium transmission

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

$$R_{0,rel} = \frac{c}{(-\ln p)} p^v$$

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 one day, and *v* is the pathogen incubation period in the mosquito. In our study, *c* was considered as the probability of a mosquito carrying *Plasmodium* DNA in its saliva. Although the presence of oocysts in mosquitoes has previously been used to determine vector competence for avian *Plasmodium* (Pigeault *et al*. 2015), here we identified the presence of parasite DNA in mosquito saliva to determine vector competence, a method widely used for assessing vectorial competence for the transmission of different pathogens (Ciota *et al*. 2017; Gutiérrez-López *et al*. 2016). In addition, we considered *v* as 13 days, following Valkiūnas (2005) and LaPointe *et al*. (2010). The relative R_0 value was calculated considering the survival rate and the proportion of mosquitoes with positive saliva samples infected with lineages of each *Plasmodium* clade.

Results

We identified four different parasite lineages in 45 infected birds, including the *Plasmodium relictum* lineages SGS1 (N=27) and GRW11 (N=6), and the lineages COLL1 (N=8) and PADOM01 (N=4). The morphospecies for COLL1 and PADOM01 is unknown, but these lineages clustered with the lineage SEIAUR01 (corresponding to *P. cathemerium,* as found in house sparrows in the same area by Ferraguti *et al*. (2018)). 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, a 0.035 uncorrected p-distance was found between lineages SGS1-GRW11 and COLL1-PADOM01. Thus, in further analyses these four lineages were grouped into two different clades: clade A corresponding to the *Plasmodium relictum* lineages SGS1 and GRW11, and clade B corresponding to the *Plasmodium spp*. lineages COLL1 and PADOM01. We found a similar parasite load in birds infected by parasites of both clades (mean ± SD: clade A: 1.29±0.21, clade B: 1.15±0.35, ANOVA; $F_{1, 38} = 0.11$, $p = 0.74$)

Parasite development in the mosquitoes

Overall, 27 and 12 *Plasmodium* infected birds were exposed to 1696 *Cx. pipiens* and 990 *Oc. caspius*, respectively. Of these, 183 (10.8%) *Cx. pipiens* and 121 (12.2%) *Oc. caspius* fed on blood. The *Plasmodium* infection status in the head-thorax was analysed for 126 *Cx. pipiens* fed on 27 infected individuals (19 infected by parasites of clade A and 8 infected by parasites of clade B) and 45 *Oc. caspius* fed on 12 infected birds (2 infected by a parasite of the clade A and 10 infected by a parasite of the 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. None of the 45 head-thoraces of *Oc. caspius* analysed showed evidence of *Plasmodium* infection.

Further analyses were restricted to *Cx. pipiens* mosquitoes, the only species with positive amplifications of *Plasmodium* DNA. A higher *Plasmodium* prevalence was found in the head-thorax of mosquitoes fed on birds with higher parasite loads (estimate (est = 0.86, $Z = 2.99$, $p = 0.003$), but parasite prevalence did not differ between clades (est = 0.42, $Z = 0.98$, $p = 0.33$). Parasites were detected in the head-thorax of 37.6% (35/93) and 48.8% (16/33) *Cx. pipiens* fed on birds infected by *Plasmodium* lineages of the clades A and B,

respectively. By contrast, a higher prevalence of clade B (21.2%, 7/33) than clade A (4.3%; 4/93) was found in *Cx. pipiens* saliva (est = 1.81, Z = 2.68, p = 0.007; Fig. 1), while non-significant associations were found with bird parasite load (est = 0.52, Z = 1.40, p = 0.16).

All *Plasmodium* lineages infecting house sparrows were isolated from mosquito saliva, supporting the competence of *Cx. pipiens* for the transmission of these four lineages. 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 166 *Cx. pipiens* fed on infected birds and 80 *Cx. pipiens* fed on 10 un-infected birds. The survival of mosquitoes did not depend on the bird infection status (Z = -1.58, p = 0.11) or the host infection intensity (Z = 1.43, p = 0.15). Mosquito age did not affect variation in survival (Z = 0.23; p = 0.81). However, when considering the identity of *Plasmodium* parasites instead of the infection status, *Cx. pipiens* fed on birds infected by parasites of the clade B survived longer than those fed on birds infected by clade A, and on uninfected birds (Z = -2.10, p = 0.04; Fig. 2). Mosquito survival was not associated with the bird parasite load (Z = 1.50, p = 0.13) neither with the mosquito age (Z = 0.19; p = 0.85)

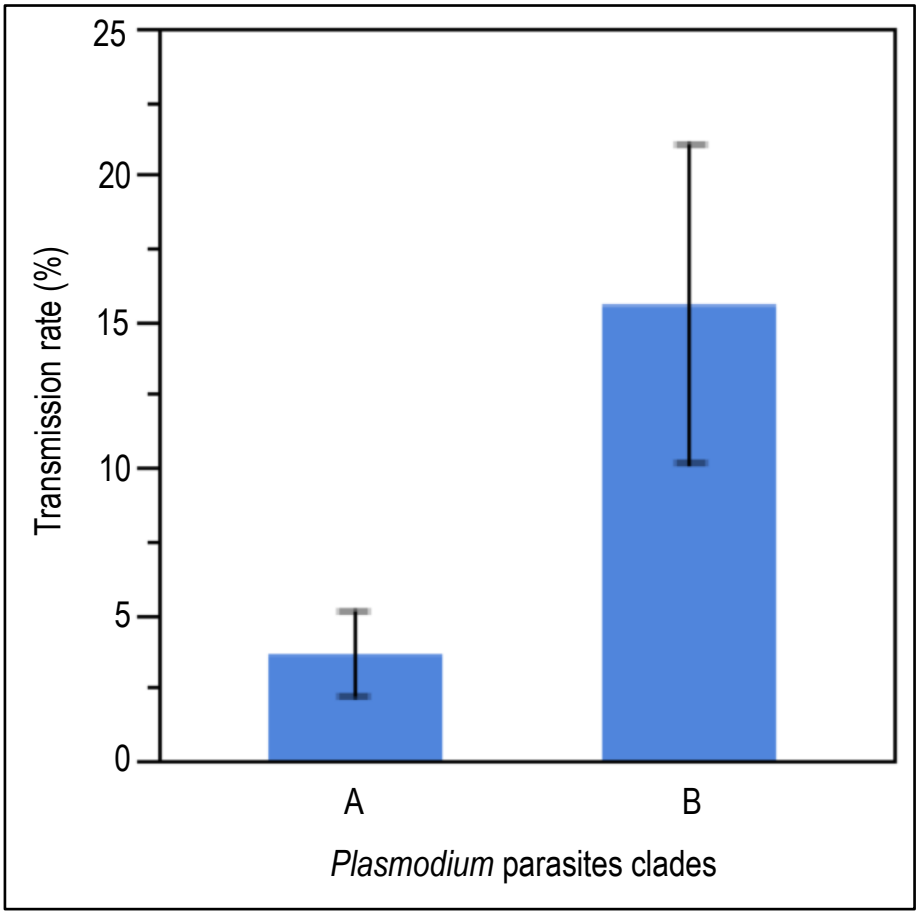

Fig. 1. Percentage of mosquito saliva with presence of *Plasmodium* DNA (i.e. transmission rate) of *Plasmodium* parasites of clade A and B by *Cx. pipiens* mosquitoes.

Plasmodium transmission risk

Both parameters, i.e. transmission rate and vector survival, were affected by the parasite clade. Consequently, *Plasmodium* parasites of the clade A showed a lower relative transmission risk number (R_0) than those of the clade B. When mosquitos fed on birds infected by *Plasmodium* parasites of the clade B, the R_0 value was 1774.6 higher than that obtained for mosquitoes fed on birds infected by *Plasmodium* parasites of the clade A.

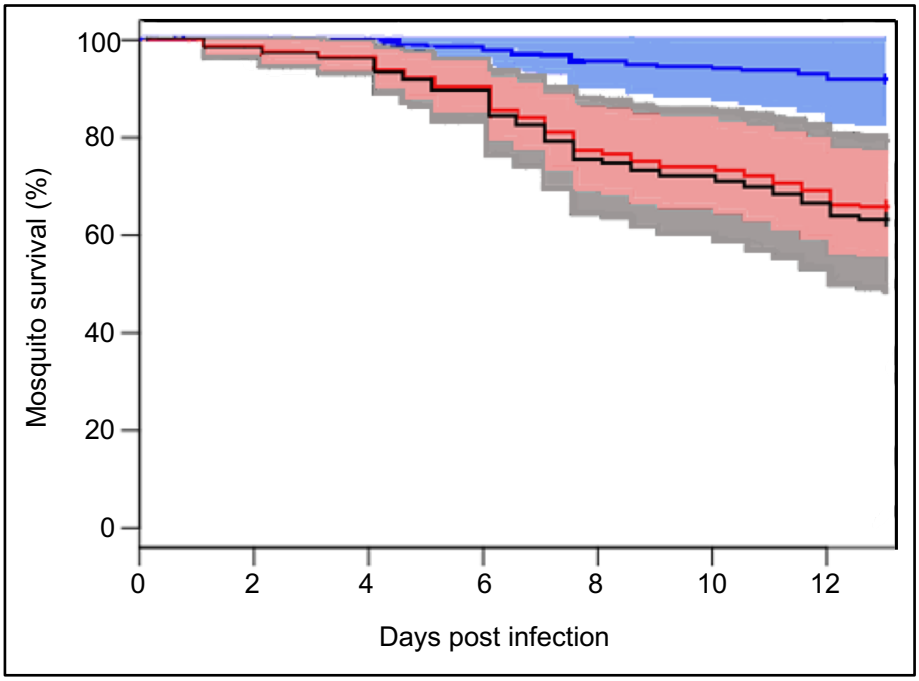

Fig. 2. Proportion of *Cx. pipiens* that survived until 13 days-post infections by *Plasmodium* parasites of clade A (red line), clade B (blue line) or control (black line). The shaded areas comprise the standard errors.

Discussion

The successful transmission of vector-borne parasites largely depends on the availability of competent mosquitoes in the area (Beerntsen *et al.* 2010). Molecular screening of avian *Plasmodium* in mosquitoes suggested the potential implication of different genera of mosquitoes in the transmission of a number of *Plasmodium* lineages (Kimura *et al*. 2010; Ferraguti *et al*. 2013; Kazlauskienė *et al*. 2013; Palinauskas *et al*. 2016; Schoener *et al*. 2017). Although mosquitoes of the genus *Culex* are considered the main vectors of avian *Plasmodium*, these parasites may develop in *Anopheles*, *Aedes* and *Lutzia* mosquitoes, which may thus play a secondary role in the transmission of these parasites (Santiago-Alarcon *et al*. 2012), suggesting that avian *Plasmodium* spp. are not tightly coevolved with mosquito species (Kimura *et al*. 2010). However, our results suggest that while the four *Plasmodium* lineages isolated here from birds could be transmitted by *Cx. pipiens*, this was not the case of *Oc. caspius* mosquitoes. The insect midgut represents a strong selective force for the parasites, being able to dramatically reduce the number of

viable parasites from those initially ingested (Hardy *et al*. 1983; Abraham *et al*. 2004). In addition, *Plasmodium* penetrates the midgut intracellular epithelium by a complex mechanism involving numerous proteins of the membrane (Han *et al*. 2000; Ishino *et al*. 2006; Siden-Kiamos *et al*. 2006; 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 *Oc. caspius* mosquitoes. Furthermore, differences in the immune response against parasites or midgut microbiota between mosquito species may affect parasite development (Azambuja *et al*. 2005; Dong *et al*. 2009; Weiss *et al*. 2011), and may partially explain our results. Clearly, our results indicate that detection of *Plasmodium* DNA in a particular mosquito species is not a good indicator of the species' vectorial competence, since *Plasmodium* DNA was previously detected, even at high prevalences, in *Oc. caspius* (Schoener *et al*. 2017).

Vector competence depends on both mosquito and parasite intrinsic factors, finally affecting the ability of a vector to transmit a pathogen (Hardy *et al*. 1983; Black *et al*. 1996; Woodring *et al*. 1996). *Culex pipiens* is considered a competent vector for different avian *Plasmodium* species (Kimura *et al*. 2010; Santiago-Alarcon *et al*. 2012; Ferraguti *et al*. 2013; Schoener *et al*. 2017). However, our results support a differential vector competence for the transmission of two clades of avian *Plasmodium*, with a higher prevalence in mosquito saliva of lineages of the clade B (COLL1-PADOM01) than for the *Plasmodium relictum* lineages of the clade A. These differences could be the result of unequal parasite development in the mosquitoes. In this respect, it is possible that both clades differ in the time required to develop and reach the salivary glands, as has been reported between parasite species (La Pointe *et al*. 2010; Palinauskas *et al*. 2016), with parasites of the clade B producing sporozoites faster than those of the clade A. In this study, mosquito saliva was obtained 13 days after parasite exposure, a period that exceeds the time needed for different *Plasmodium* spp. to develop in the mosquito salivary glands (Valkiūnas 2005; LaPointe *et al* 2010). Supporting this possibility, *Plasmodium relictum* sporozoites in the salivary glands of mosquitoes have been recorded as early as 4 and 5 days post infection (Rosen & Reeves, 1954; Work *et al*. 1990). In addition, Kazlauskienė *et al.* (2013) identified the presence of sporozoites of the *Plasmodium relictum* lineages SGS1 and GRW11 (corresponding to those of the clade A in this study) 14 days post infection in the salivary

glands of *Cx. pipiens* mosquitoes (yet mosquitoes at 13 dpe were not analysed). In addition to parasite identity, the parasite load of the donor host may largely determine the success of parasite development in the insect vector and, potentially, its capacity for parasite transmission. In humans, *Plasmodium* gametocytaemia was positively associated with the mosquito infection rates (Jeffery & Eyles, 1955; Bousema & Drakeley, 2011), while non-significant associations between the parasitaemia of avian *Plasmodium* and the oocyst prevalence were found (Pigeault *et al*. 2015). Our results suggest that *Plasmodium* load in the avian host determine the infection rate in mosquitoes, although its importance for the final development of parasites in mosquito saliva may be modulated by other factors, including specific mosquito-parasite assemblages.

The costs of *Plasmodium* infection in mosquito survival remain a subject of intense debate (Ferguson & Read, 2002). Vézilier *et al*. (2012) reported 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 failed to find any significant difference in mosquito survival when both mosquitos fed on infected birds were compared with those fed on uninfected individuals. However, clear differences were found when parasite identity was considered in the analyses, with mosquitoes fed on birds infected by *Plasmodium* clade B showing a 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. However, a differential impact on bird hosts of parasite lineages/morphospecies have been reported. For instance, Lachish *et al*. (2011) found that *P. relictum* had a lower virulence on birds than *P. circumflexum*, supporting that different avian malaria species can have very different effects on fitness components in a single host species. Our results suggest that this may also occur in mosquitoes, with differential cost (i.e. energetic cost; Hurd *et al*. 2005) imposed by different species/lineages of *Plasmodium*. Further studies should include sevaral species of *Plasmodium* to determine the existence of potential differences in parasite virulence.

We found that *Plasmodium* transmission risk differed between parasite clades mainly due to their differential impact on mosquito survival and transmission rate. Lineages

of *P. relictum* (clade A) had a higher virulence on mosquitoes and also showed a lower transmission rate (i.e. presence in saliva) than parasites of the 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). Interestingly, *Plasmodium 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, generalist behaviour has been associated with reduced parasite fitness, due to a reduction of the replication/transmission of the parasite in novel hosts, as well as the reduction of fitness in the original hosts (Benmayor *et al*. 2009). Therefore, specialized parasites, with a limited host range, may replicate faster, being more effectively transmitted than generalist (less virulent) parasites (Leggett *et al*. 2013). Our results showed a higher efficacy of transmission from infected house sparrows to *Cx. pipiens* in the case of clade B as compared with clade A. This could be due to the generalist character of *P. relictum*, which may decrease its fitness when transmitted by *Cx. pipiens*, but not when infecting birds (Hellgren *et al*. 2009).

In conclusion, results from this study confirm the existence of inter- and intra-specific differences in the ability of *Plasmodium* lineages to develop in mosquito species. While some mosquitoes such as *Oc. caspius*, completely limit the parasite development, *Cx. pipiens* may play a key role in the transmission of avian *Plasmodium*. Nevertheless, the identity of each vector-parasite assemblage may modulate the transmission success of *Plasmodium* lineages through differences in parasite development rate in the mosquito and the costs of infection on mosquito survival.

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Chapter 5

Infection load influence *Plasmodium* transmission risk due to their effects on mosquito survival

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Abstract

Plasmodium transmission success depends on a trade-off between the use of host resources to favour parasite reproduction and the negative effects on host health, which can be mediated by infection intensity. Despite its potential influence on parasite dynamics, the effects of infection intensity in birds and vectors on *Plasmodium* transmission risk is still poorly understood. Here, we experimentally reduced the *Plasmodium* load in naturally infected wild house sparrows to assess how this would affect *Plasmodium* transmission. We monitored the lifespan of *Culex pipiens* mosquitoes and their infection status by analysing the head-thorax and saliva at 13 days post-exposure in Primaquine-medicated and infected-control birds. We found that the proportion of mosquitoes infected by *Plasmodium* and the presence of *Plasmodium* in saliva were not associated with the treatment designed to reduce infection intensity in birds. However, the mosquitoes that fed on medicated birds showed a basic reproduction number (R_0) 19.66 times higher than those that fed on control birds due to the negative impact of the parasite load on vector survival. We thus suggest that high *Plasmodium* infection intensities in hosts may be negatively selected due to a reduction in vector survival. Consequently, the transmission risk of avian *Plasmodium* will be higher during the chronic phase of infection in birds than during the acute phase of infection.

Introduction

Parasites depend on their hosts to survive and to maximise their fitness (de Roode *et al*. 2008). Vector-borne parasites such as *Plasmodium*, require two different hosts to complete their life cycles; a vertebrate host for asexual replication and an insect vector for sexual reproduction and the development of sporozoites (Valkiūnas, 2005). These processes occur in two phylogenetically distant organisms and give rise to complex interactions between hosts, vectors and parasites, as well as promoting constant coevolution between them (Ewald, 1983; Cohuet *et al*. 2010). Avian *Plasmodium* infection is characterised by an acute phase, in which high parasite loads are reached soon after infection, followed by a chronic phase characterised by lower intensities that usually continue their course as lifelong infections (Asghar *et al*. 2012). Greater infection intensity has often been associated with higher transmission rates (Mackinnon & Read, 1999; Griffin *et al*. 2010). However, a rapid replication rate could reduce the transmission success of the parasite by killing either its vertebrate or invertebrate host (Mackinnon & Read, 1999). Parasite virulence may thus be considered as a balance between increasing parasite transmission and reducing the costs imposed on their hosts (de Roode *et al*. 2008).

The basic reproduction number (R_0) –the average number of secondary infections caused by an infectious individual in a naïve population (Smith *et al*. 2012) – is widely used to understand the epidemiology of the pathogens that cause malaria (Parham & Michael, 2010), Lyme (Hartemink *et al*. 2008) or bluetongue diseases (Gubbins *et al*. 2008). R_0 is estimated using different parameters of the disease system in question, some of which such as the mosquito survival rate are particularly important (Ross, 1911; Macdonald, 1955). Mosquitoes must be able to survive long enough for the *Plasmodium* sporozoites to develop (7–13 days; Macdonald, 1952; Valkiūnas, 2005) to guarantee subsequent parasite transmission. Therefore, the lifespan of infected mosquitoes will have drastic consequences for the *Plasmodium* transmission risk (Garrett-Jones & Grab, 1964; Smith *et al*. 2012). However, to the best of our knowledge, the effects of *Plasmodium* infection on pathogen R_0 remain untested empirically.

Although the impact of *Plasmodium* survival on birds has been experimentally demonstrated (Martínez-de la Puente *et al*. 2010; Asghar *et al*. 2015), much less is known about the effects of infection on vector survival (Ferguson & Read, 2002). Mosquito

survival may be reduced by *Plasmodium* due to tissue damage during the development and migration of parasites from the midgut to the salivary glands (Vaughan & Turell, 1996). Indeed, positive (Vézilier *et al*. 2012), negative (Lalubin *et al*. 2014) and no effects (Pigeault & Villa, 2018) of the infections by avian *Plasmodium* on mosquito survival have all been reported. Moreover, infection intensity in the hosts may determine the successful development of the parasite in the mosquito (Griffin *et al*. 2010; Bousema & Drakeley, 2011), although this relationship is not linear in the case of *Plasmodium* falciparum (Churcher *et al*. 2013). In avian *Plasmodium*, Pigeault *et al*. (2015) found that the successful development of oocysts in mosquitoes, which may be considered as a proxy for transmission capacity, did not depend on the host infection intensity.

Here, we used birds naturally infected by avian *Plasmodium* to experimentally test the effect of host infection intensity on mosquito infection and survival. *Culex pipiens*, the main vector of avian *Plasmodium*, were allowed to bite *Plasmodium*-infected birds that had been either medicated with the antimalarial Primaquine, which reduces infection intensity, or non-medicated control birds. The impact of this treatment on the *Plasmodium* transmission risk was quantified by estimating the R0 for each experimental group.

Material and methods

Mosquito collection and rearing

Culex pipiens larvae were collected in July 2014 in the natural reserve La Cañada de los Pájaros (Seville, Spain; 6°14´W, 36°57´N). Larvae were transferred to the laboratory and maintained in fresh water in plastic trays at uniform density, and fed ad libitum (Mikrozell 20ml/22g; Dohse Aquaristik GmbH & Co. KG, D-53501, Gelsdorf, Germany). Larvae and adult mosquitoes were maintained at 28±1 ºC, 65–70% Relative Humidity and 12:12h light:dark cycle. Adult mosquitoes were anesthetised with ether, sexed and identified to species level under a stereo-microscope (Nikon SM7645) on chilled Petri dishes using morphological keys (Schaffner *et al*. 2001). Female mosquitoes were placed in insect cages (BugDorm-43030F, 32.5×32.5×32.5 cm) and fed ad libitum with 1% sugar solution. Sugar solution was replaced with water 24h prior to each experiment and the water was removed from the cages 12 hours before the experiment begins. The experiment was conducted using 7–15-day-old female mosquitoes.

Bird trapping and sampling

Yearling house sparrows (*Passer domesticus*) were captured using mist nets in July 2014 (6°50' W, 37°18' N). Birds were individually ringed and blood was sampled (0.2 ml) from the jugular vein using sterile syringes to assess their *Plasmodium* infection status using molecular methods (see details below). Birds were transported to the laboratory in the Doñana Biological Station (EBD-CSIC) and kept in birdcages (58.5×25×36 cm) in a vector-free room under controlled conditions (22±1 °C, 40–50 % RH and 17:7 h light:dark cycle). Birds were housed for two weeks before the start of the experiments and were fed ad libitum with a standard mixed diet for seed-eating and insectivorous birds (KIKI, GZM S.L., Alicante, Spain).

Experimental procedure

The birds' infection statuses were determined by the amplification and sequencing of a fragment of the parasite cytochrome b gene (*cyt b*) following Hellgren *et al*. (2004), see details below. Thirty-six house sparrows, naturally infected by *Plasmodium*, were randomly assigned to one of two experimental groups: medicated birds (the experimentally reduced infection intensity group, N=17) or control birds (non-medicated group, N=19). Medicated birds were injected subcutaneously with 0.1mg of the antimalarial drug Primaquine (Sigma, St. Louis, MO, USA) diluted in 0.1ml saline solution while control birds were injected subcutaneously with the same volume of saline solution (see Yan *et al*. 2017). Primaquine was previously used to reduce the intensity of infection by avian malaria and malaria-like parasites in different bird species, including house sparrows (Merino *et al*. 2000; Martínez-de la Puente *et al*. 2010; García-Longoria *et al*. 2015). In vertebrates, high doses of Primaquine produces non-desirable side effects, such as gastrointestinal disturbances and the development of methaemoglobinaemia (Mayorga *et al*. 1997). Thus, only a single and low–concentration dose of Primaquine was administered to minimize these side effects. A single dose will clear most of the gametocytes within seven days after treatment, as reported in humans (Burgess & Bray, 1961). Ten days after the treatment, each bird in the two experimental groups was immobilized (using a cylinder of 1×1 cm mesh, allowing mosquitoes can bite through) and exposed to 80 unfed female *Cx. pipiens*

in insect cages (BugDorm-43030F 32.5×32.5×32.5 cm) for 30 minutes. Immediately after the trials, engorged mosquitoes were captured, placed in insect cages under standard conditions (28±1 ºC, 65-70% RH and 12:12 light: dark cycle) and fed ad libitum with 1% sugar solution. Mosquito survival was monitored every 12h for 13 days post-exposure to allow for parasite development. At the end of this period, saliva from the surviving mosquitoes was obtained by introducing the mosquitoes' proboscis into a 1µl disposable capillary (Einmal-Kapillarpipetten, Hirschmann® Laborgeäte, Germany) with 1 µl of fetal bovine serum. One µl of 2% pilocarpine (Novartis 2012, Alcon Cusí S.A. Barcelona, Spain) was applied to the mosquito thorax to stimulate salivation. After 45 min, the medium containing the saliva was placed in 1.5 ml Eppendorf tubes with 10 µl of MQ water (see details in Gutiérrez-López *et al*. 2016). Samples were kept at -80 ºC until further molecular analyses.

One day after the trial, the birds' blood was sampled again (0.2 ml) to confirm the blood parasite lineages infecting individuals at this stage. This procedure allowed us to identify any potential parasite lineage that was not detected during the first sampling. After sampling, a drop of blood was immediately smeared, air-dried, fixed in absolute methanol and stained with Giemsa for 45 min (Gering and Atkinson, 2004). The intensity of infection by haemosporidians parasites was estimated as the percentage of infected red blood cells detected after scanning 10,000 erythrocytes from each blood smear at high magnification (x10,000). Birds were not blood-sampled immediately before or during the mosquito exposure period in order to reduce the stress caused by the blood extraction and mosquito bites. Birds were released after the completion of the experiments at the site of capture.

Molecular detection and identification of blood parasites

DNA was isolated from blood samples and the head-thorax of each mosquito using a semiautomatic procedure (MAXWELL® 16 LEV Blood DNA Kit; Gutiérrez-López *et al.* 2015). The Qiagen DNeasy® Kit Tissue and Blood (Qiagen, Hilden, Germany) was used to isolate DNA from mosquito saliva. Based on a previous study showing that saliva from uninfected mosquitoes tested negative (Gutiérrez-López *et al.* 2016), we only analysed those saliva samples from mosquitoes with *Plasmodium* positive head-thoraxes. *Plasmodium* infections were recorded following Hellgren *et al*. (2004). The presence of

amplicons was verified in 1.8% agarose gels and positive samples were sequenced using the Macrogen sequencing service (Macrogen Inc., Amsterdam, The Netherlands). Sequences were edited using the software Sequencher™ v 4.9 (Gene Codes Corp., © 1991–2009, Ann Arbor, MI 48108) and assigned to parasite lineages through blast comparison with those deposited in the GenBank database (National Center for Biotechnology Information) and Malavi (Bensch *et al*. 2009).

Statistical analyses

An ANOVA test was used to assess differences in the *Plasmodium* infection intensity between medicated and control birds. We fitted a Cox mixed-effect model by maximum likelihood to mosquito survival data (number of surviving mosquitoes /12-hours-period) to test the effect of the medication treatment on mosquito survival. The medication treatment was considered as a fixed factor and bird identity as a random or 'frailty' effect. Two similar Generalized Mixed Linear Models (GLMMs) with binomial error and logit link function were performed in which the infection status by *Plasmodium* of the head-thorax or the saliva samples were included as the dependent variable, respectively. The medication treatment was included as a fixed factor and bird identity as a random term. Mosquitoes that fed on birds with coinfections (N=51 mosquitoes fed on 6 birds: 2 medicated, 4 controls) were removed from the analyses to avoid any potential confounding effects of co-infections on mosquito survival and the presence of *Plasmodium* DNA in the mosquito saliva (see Marzal *et al*. 2008). Statistical analyses were performed in R software 3.2.5 (R Core Development Team, 2016) with the packages survival (Fox, 2002) and lme4 (Bates *et al*. 2015).

Estimation of the impact on Plasmodium transmission

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

$$R_{0,rel} = \frac{c}{(-\ln p)} p^v$$

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 one day, and v is the pathogen's mosquito incubation period. In our study, c was considered as the probability of a mosquito carrying *Plasmodium* DNA in its saliva. Although the presence of oocysts in the mosquitoes has previously been used to determine vector competence for avian *Plasmodium* (Pigeault *et al*. 2015), here we identified the presence of parasite DNA in mosquito saliva, a method widely used in studies of vector competence for the transmission of different pathogens (Ciota *et al*. 2017; Gutiérrez-López *et al*. 2016). In addition, we considered v as 13 days, following Valkiunas, (2005) and LaPointe *et al*. (2010). The relative R_0 value was calculated considering the survival rate and the proportion of mosquitoes with positive saliva samples in each experimental group. See Electronic Supplementary Material for further details of this procedure.

Results

The Primaquine treatment significantly reduced the infection intensity in medicated birds compared to the controls (mean±SE control = 1.69±0.26, medicated =0.79±0.23, $F_{1,35}$= 5.77, p= 0.02). The mosquitoes that fed on medicated birds (N= 102) had a higher daily survival probability than those that fed on controls (N= 95) (probability=0.99 and 0.96, respectively, Z = -3.17, p = 0.002; Table 1; Fig. 1). The presence of *Plasmodium* in the head-thorax of surviving mosquitoes was evaluated in 76 and 46 individuals that fed on medicated and control birds, respectively (Table 1). The medication treatment did not affect the proportion of mosquitoes with *Plasmodium*-positive head-thorax (est = 0.89, Z = 0.92, p = 0.36, Table 1) or saliva (est = 1.31, Z = 1.66, p = 0.10, Table 1).

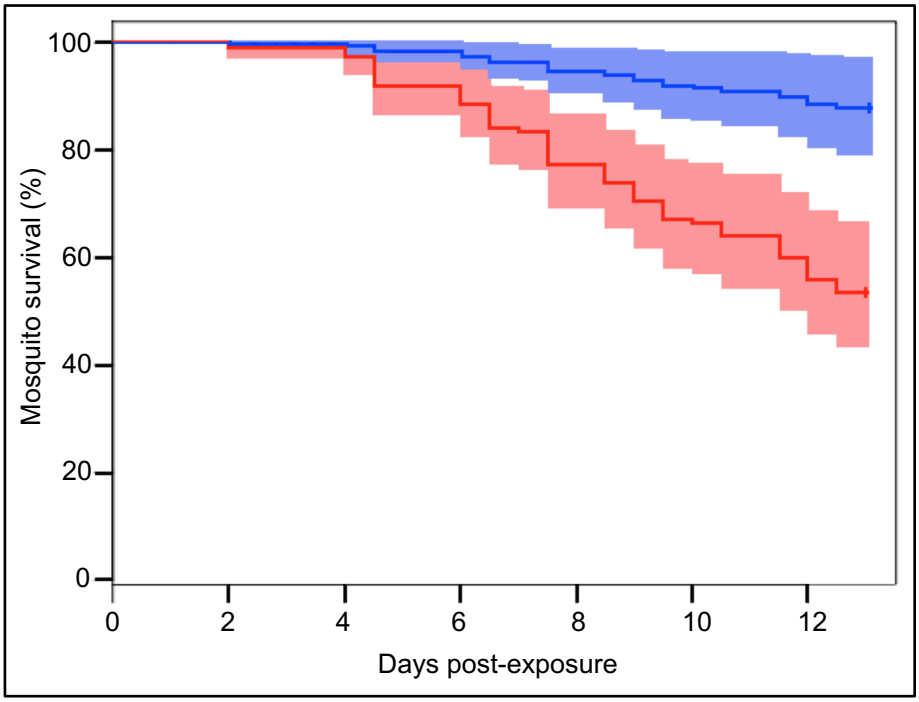

Fig. 1. Percentage of mosquitoes' survival until 13 days-post exposure to Primaquine-medicated (blue) and control birds (red). The shaded areas comprise the standard errors.

Table 1. Number of engorged, surviving and analysed *Culex pipiens* mosquitoes for the two experimental groups of birds (i.e. medicated and control). The number of *Plasmodium* positive/analysed head-thorax and mosquito saliva is given for each group. *Three mosquitoes fed on control birds and four mosquitoes fed on medicated birds escaped and were not included in survival analyses. **Three mosquitoes fed on control birds and ten mosquitoes fed on medicated birds were not analyzed due to logistical problems

Treatment	Engorged mosquitoes*	Alive mosquitoes 13 days post exposure	Analysed mosquitoes**	Positive Head-thorax	Positive Saliva
Control	95	49	46	12	2
Medicated	102	86	76	31	11

The reduced infection intensity associated with the medication treatment had a large impact on the *Plasmodium* R_0 due to the negative effects on the survival of mosquitoes that fed on control birds; the R_0 for mosquitoes biting medicated birds was 19.66-times higher than those that feeding on control birds.

Discussion

The epidemiology of vector-borne parasites depends on a number of factors affecting the transmission risk such as host density (Gubbins *et al*. 2008), host recovery rate (Macdonald, 1955), vector density (Hartemink *et al*. 2011), and temperature (Parham & Michael, 2010). We experimentally manipulated the infection intensity in wild birds in order to assess the impact this would have on the *Plasmodium* transmission risk via effects on mosquito lifespan and infection rate. Medication reduced the infection intensity in birds, which in turn influenced mosquito survival since higher *Plasmodium* intensities gave rise to greater mortality rates. Consequently, mosquitoes that fed on medicated birds had a higher *Plasmodium* R_0 than those that fed on control birds. The alternative interpretation – i.e. that the greater survival rates in the mosquitoes that fed on medicated birds was in fact due to an effect of the drug itself on mosquito survival – is implausible as the biological half-life of Primaquine in plasma is 4–9 h (Baird & Hoffman, 2004), and mosquitoes fed on the birds 10 days after medication. Even if the drug had been active when ingested by the vectors, it could have not favoured mosquito survival (Butcher, 1997).

The costs of *Plasmodium* infection on mosquito survival are still a subject of intense debate (Ferguson & Read, 2002). Vézilier *et al*. (2012) report increased longevity in *Culex pipiens* infected by *P. relictum*; Pigeault and Villa (2018), in contrast, found that there were no effects on mosquito survival when using the same mosquito–parasite assemblage. However, these effects could be driven by the access to nutritional resources other than blood, and the cost of *Plasmodium* infection on mosquito survival may only be detected in the event of nutritional stress (Lalubin *et al*. 2014). The consumption of glucose has been found to be higher in infected mosquitoes than in uninfected ones (Hurd *et al*. 2005), which could be associated with the increased resources required by mosquitoes to fight off infections (Ahmed & Hurd, 2006). Parasites may impose additional costs on mosquitoes by producing tissue damage during their development, thereby increasing their susceptibility to bacterial infections and other diseases (Vaughan & Turell, 1996).

The infection intensity by *Plasmodium* in the bird host might determine the success of parasite development in the insect vector and, consequently, its parasites transmission ability. In humans, the density of *Plasmodium* gametocytes seen in blood has been found to be positively associated with the proportion of mosquitoes harbouring oocysts (Bousema

& Drakeley, 2011). However, Pigeault *et al*. (2015) failed to find any association between avian *Plasmodium* infection intensity and the probability of mosquito infection. The absence of significant associations between the experimental reduction of *Plasmodium* infection intensity and the proportion of infected mosquitoes reported here could be due to the ability of *Plasmodium* to develop in mosquitoes that have fed on vertebrate hosts with infection intensities that are low or undetectable by microscopy (Churcher *et al*. 2013; Lin *et al*. 2014). This may also explain the absence of any significant effect of the reduction of infection intensity in the presence of *Plasmodium* in mosquito saliva 13 days after blood ingestion, a period that exceeds the time required for *Plasmodium* to develop in the salivary glands (Valkiūnas, 2005; LaPointe *et al*. 2010).

In wild bird populations, infections by avian *Plasmodium* usually typically cause an acute phase of infection with high infection intensities followed by a chronic phase with low infection intensities (Asghar *et al*. 2012). According to our results, *Plasmodium* transmission may be more effective during the chronic phase of infection than during the acute phase due to the negative effects of high infection intensities on mosquito survival. Interestingly, Cornet *et al*. (2013) found that mosquitoes prefer biting avian hosts in a chronic phase of infection to biting uninfected birds or birds with infections in an acute phase, which provides further support for our results on how chronically infected birds affect the epidemiology of avian *Plasmodium*.

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Supporting Information Legends

SI 1. *Estimation of the Plasmodium relative basic reproduction number* ($R_{0,\,rel}$)

The relative R_0 of *Plasmodium* transmitted by *Culex pipiens* to birds was estimated using a simplified equation of the R_0 epidemiology model derived from the equation proposed by Macdonald (1955) for human *Plasmodium*:

$$R_0 = \frac{m * a^2 * b * c}{(-\ln p)\, r} p^v$$

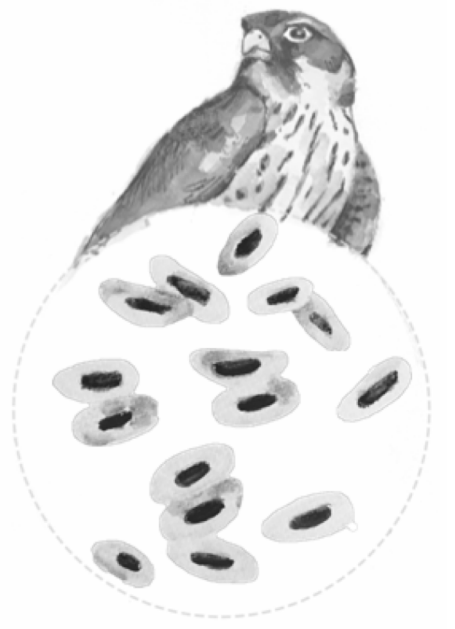
where m is the ratio of mosquito to birds; a is the feeding rate of mosquitoes; b the proportion of bites by infectious mosquitoes that infect a vertebrate host; c the probability a mosquito becomes infected after biting an infected vertebrate host; p is the daily survival rate; r the daily rate that birds recovers from infections; and v is the pathogen's mosquito latent period. The variables m, a, b and r were considered constants in our calculations of R_0, which gave a simplified formula that allowed us to estimate the impact of changes in the probability of infection after feeding on an infected bird and of mosquito survival on avian *Plasmodium* transmission:

$$R_{0,rel} = \frac{c}{(-\ln p)} p^v$$

SI 2. *Avian Plasmodium lineages found in birds*

The mosquitoes that fed on six birds that showed evidence of co-infection by more than one *Plasmodium* lineage were excluded from the experiments. Overall, four different parasite lineages were identified in birds: the *Plasmodium relictum* lineages SGS1 (N=21, medicated = 12, control = 9) and GRW11 (N=5, medicated = 1, control = 4), and the lineages PADOM02 (N=3, medicated = 2, control = 1) and COLL1 (infecting a control bird). *Plasmodium* lineages isolated from the head-thorax of mosquitoes and their saliva were identical. With the exception of the *Plasmodium* lineage COLL1, all *Plasmodium* lineages infecting house sparrows were isolated from mosquito saliva, supporting the competence of *Cx. pipiens* for the transmission of these lineages.

Section 3
Vector-Host-Pathogen Interactions



Chapter 6

Low prevalence of blood parasites in a long-distance migratory raptor: the importance of host habitat

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Abstract

The low prevalence of blood parasites in some bird species may be related to the habitats they frequent, the inexistence of the right host-parasite assemblage or the immunological capacity of the host. Here, we assess the parasite load of breeding populations of Eleonora's falcon (*Falco eleonorae*), a medium-sized long-distance migratory raptor that breeds on small isolated islets throughout the Mediterranean basin and overwinters in inland Madagascar. We examined the prevalence and genetic diversity of the blood parasites belonging to the genera *Plasmodium*, *Haemoproteus* and *Leucocytozoon* in Eleonora's falcon nestlings from five colonies and in adults from two colonies from nesting sites distributed throughout most of the species' breeding range. None of the 282 nestlings analysed were infected by blood parasites; on the other hand, the lineages of *Plasmodium*, *Haemoproteus* and *Leucocytozoon* were all found to infect adults. Our results support the idea of no local transmission of vector-borne parasites in marine habitats. Adult Eleonora's falcons thus may be infected by parasites when on migration or in their wintering areas. The characteristics of marine environments with a lack of appropriate vectors may thus be the key factor determining the absence of local transmission of blood parasites. By comparing the parasite lineages isolated in this species with those previously found in other birds we were able to infer the most likely areas for the transmission of the various parasite lineages.

Introduction

The presence and abundance of insect vectors is a key factor affecting the interaction between blood parasites and wild bird populations (Sol *et al.* 2000; Martínez-de la Puente *et al.* 2013). Indeed, habitat characteristics influence both birds' habitat choice during the breeding season and the viability of insect vector populations and may ultimately determine the success of blood parasite transmission (Mendes *et al.* 2005). Piersma (1997) has suggested that bird species inhabiting marine habitats such as small isolated islets or sea cliffs usually have lower blood parasite prevalence than species inhabiting inland areas (i.e. mainland and/or large islands) due to the scarcity of insect vectors in marine habitats. Marine habitats whose environments are characterized by high salinity, exposure to winds and a lack of vegetation cover are generally unsuitable places for insect vectors that require an aquatic larval stage to complete their life-cycles (Super & van Riper 1995). In fact, a number of studies on seabirds have found a low prevalence or total absence of blood parasites and the suggested cause is the scarcity of insect vectors (Piersma, 1997; Martínez-Abraín *et al.* 2002; Jovani *et al* 2001). However, in addition to the role of vectors, other factors such as the existence of the right host-parasite assemblage and/or the immunological capacity of the avian host to fight off infections may also affect the outcome of host-blood parasite interactions (Mendes *et al.* 2005; Martínez-Abraín *et al*. 2002). Migratory bird species that use a range of habitats throughout their life-cycles (e.g. marine and freshwater inland habitats) are excellent study models for exploring the relative importance of the potential mechanisms involved in parasite transmission.

In this study, we assessed variation in blood parasite prevalence between breeding colonies and host status (nestlings vs. breeding adults) in Eleonora's falcon (*Falco eleonorae*). This long-distance migratory raptor breeds colonially on small isolated islets throughout the Mediterranean basin and overwinters in inland Madagascar (Walter, 1979; Gschweng *et al.* 2008; López-López *et al.* 2009). We used a PCR-based approach to determine the prevalence and the genetic identity of three avian blood parasite genera, *Plasmodium*, *Haemoproteus* and *Leucocytozoon*, that potentially infect these falcons. These parasite genera commonly infect birds and all have similar life cycles that require the presence of haematophagous insect vectors if they are to be transmitted (Valkiūnas, 2005).

In the light of Piersma's hypothesis (Piersman, 1997), we expected to find a general paucity of blood parasites in Eleonora's falcons in marine habitats. To determine the relative importance of habitat-related *vs*. host-related mechanisms on parasite transmission, we 1) compared the parasite load in nestlings from five different breeding colonies located in sites scattered throughout most of the species' breeding range (Fig. 1) to determine whether or not local transmission of vector-borne blood parasites occurs on marine breeding grounds, and 2) compared the blood parasite prevalence in nestlings with that of adults from two of these breeding areas. Unlike nestlings, adult Eleonora's falcons are exposed during their annual cycle to a huge range of habitats and their associated pathogens.

Material and methods

The vertebrate host

Eleonora's falcon breeds colonially in marine environments, mainly on the sea cliffs of small islands and rocky islets in the Mediterranean Basin (from Spain to Greece), as well as on several islets in the eastern Atlantic Ocean (the Canary archipelago and the Îles Purpuraires off the north coast of Africa) (Walter, 1979). When raising offspring, this species is highly specialized in the hunting of migratory birds that are heading to Africa. Accordingly, breeding colonies are strategically situated on small islands and islets located along the main migratory flyways. This falcon has a delayed breeding season and is the tardiest breeder (August–October) of all Northern Hemisphere raptor species (Gangoso *et al.* 2013). They lay a single clutch of 1–4 eggs. Incubation lasts for 31 ± 2 days and nestlings fledge at 35–40 days (Wink *et al.* 2000). After breeding, Eleonora's falcons undertake a long-distance migration across continental Africa to their winter quarters in Madagascar (López-López *et al.* 2009). This migratory journey takes about 1–2 months and passes through at least 12 countries where they perform several stopovers (López-López *et al.* 2009). In winter, Eleonora's falcons shift both their choice of food items (from birds to insects) and habitat and occupy humid areas of northern-central Madagascar, where the high rainfall can lead to an abundance of insects (Mellone *et al.* 2012a).

Study area and blood sampling

We sampled five Eleonora's falcon populations located in three different countries in the Mediterranean Basin (Figure 1) in 2008–2012. These populations were selected to cover most of the species' breeding range, from the westernmost (Alegranza Islet, Canary Islands, Spain) to the easternmost (Andros islet, Cyclades Islands, Greece) breeding sites (Figure 1). All birds were measured, bled and released after handling. Wing length (mm) was used to estimate nestling age (±1 day) as per Ristow and Wink (2004). When nestlings were 25–28 days old, a blood sample (0.2 ml) was extracted with a syringe from the brachial vein. In a previous study, it was found that 16–23-days old sparrowhawk (*Accipiter nisus*) nestlings were infected by *Haemoproteus* and *Leucocytozoon* (Svobodová *et al.* 2015). This supports the idea 25 days is enough time for blood parasites to be detected in the peripheral blood of Eleonora's falcon nestlings. In addition, the adult Eleonora's falcons, i.e., individuals that had performed at least one complete migration and had overwintered in inland Madagascar, from both Illa Grossa and Alegranza, were trapped using dhogaza nets and a stuffed eagle owl (*Bubo bubo*) as a decoy. Adult birds were bled in the same way as nestlings. Blood samples were preserved in absolute ethanol and stored at −20°C until molecular analysis.

DNA extraction and blood parasite analyses

Genomic DNA was isolated from blood samples using a standard chloroform/isoamyl alcohol method (Ferraguti *et al.* 2013a). A 478 bp fragment of the mitochondrial cytochrome b gene of blood parasites was amplified as per Hellgren *et al.* (2004). The presence of amplicons was verified in 1.8% agarose gels. All negative samples in a first screening were repeated twice to minimize the possibility of false negatives. Positive samples obtained using the standard chloroform/isoamyl alcohol method were reextracted using the Qiagen DNeasy® Kit Tissue and Blood (Qiagen, Hilden, Germany) and a further PCR was performed to identify blood parasites lineages.

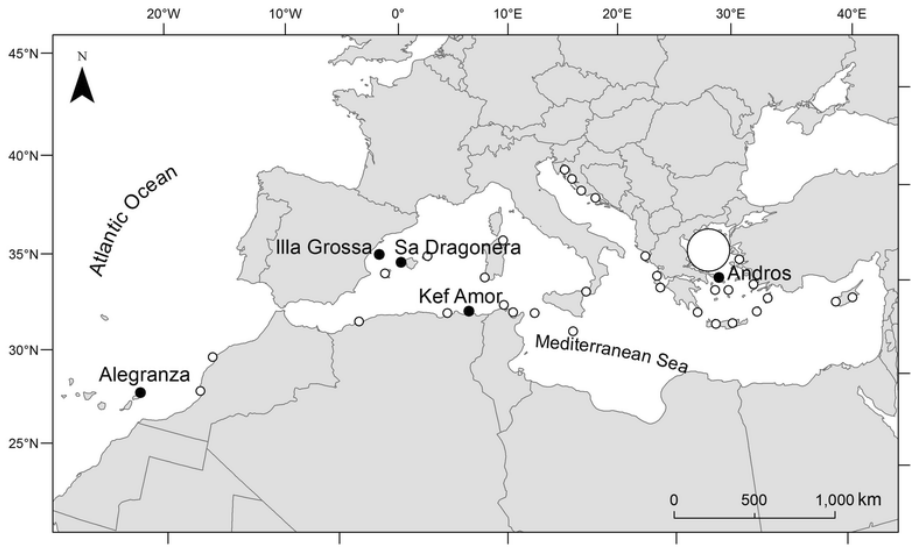

Fig 1. Map of the entire breeding range of Eleonora's falcon. Solid black circles show the colonies where blood sampling was carried out for this study. The other colonies of the species are represented by white circles.

We used this second step for sequencing because the quality of DNA sequences – but not the amplification success – significantly improves using this commercial method when compared to the standard chloroform/isoamyl alcohol method (Gutiérrez-López *et al.* 2015). Sequencing reactions were performed according using the BigDye technology (Applied Biosystems) and sequenced in both directions through a 3130xl ABI automated sequencer (Applied Biosystems). The primers HaemF and HaemR2 for *Plasmodium* and *Haemoproteus* genera and HaemFL and HaemR2L for *Leucocytozoon* genus were used. Sequences were edited using the SequencherTM v4.9 software (Gene Codes Corp., © 1991–2009, Ann Arbor, MI 48108). Parasite lineages were identified by comparison with sequences deposited in GenBank (National Center for Biotechnology Information, Blast, 2008). Blood parasite prevalences in adults and nestlings from Alegranza, the only locality where blood parasites were detected (see results), were compared using Chi-square tests (Statistica V. 7.0, StatSoft, I.N.C. 2001).

Ethical approval details

Corresponding permissions were issued by the Spanish, Algerian, and Greek Regional Administrations, according to National laws. Specific permissions numbers: MAOT N° 11908, MAOT N° 6468, MAOT N° 9723, E-87-10-T, E-59-11-E, and 95144/42.

Results

Out of the total of 324 individuals sampled (282 nestlings and 42 adults, see Table 1), blood parasite infections were only found in seven adult falcons (7/42; prevalence in adults = 16.7%), all from the Alegranza population (adult intrapopulation prevalence = 20.0%). None of the nestlings analysed had blood parasites. Parasite infection differed significantly between age classes in Alegranza, the only population where infections were detected (adults: 7/35, nestlings: 0/173; $\chi 2 = 29.92$, d. f. = 1, $P < 0.0001$). Overall, we found four different genetic lineages infecting adult Eleonora's falcons: two *Haemoproteus* lineages (lineage LK4, which was isolated from three adults, and lineage hBUBIBI01, which was isolated from a single individual); *Plasmodium* lineage LK6 (isolated from two adults); and a single individual infected by *Leucocytozoon* lineage L_CIAE02. None of the adults showed any evidence of infection by more than one parasite lineage.

Table 1. Summary of the Eleonora's falcon breeding populations and sample sizes used in this study.

Age	Locality	Infected/Sample	Parasite lineages (number of infected birds)
Nestlings			
	Alegranza	0/173	
	Illa Grossa	0/36	
	Sa Dragonera	0/11	
	kef Amor	0/44	
	Andros islet	0/18	
Aduts			
	Alegranza	7/35	Haemoproteus LK4 (3), Haemoproteus hBUBIBI01 (1), Plasmodium LK6 (2), Leucocytozoon L_CIAE02
	Illa Grossa	0/7	

Discussion

We found that none of the Eleonora's falcon nestlings from any of the populations in the Mediterranean basin was infected by blood parasites; on the other hand, 20.0% of the adults from the Alegranza population were infected by at least one of the blood parasite genera identified (Table 1). However, the overall prevalence of each blood parasite lineage infecting the adults in this population was very low, ranging from 2.86% (*Leucocytozoon* L_CIAE02) to 8.57% (*Haemoproteus* LK4). The absence of parasite infections in adults from Illa Grossa and from nestlings from Sa Dragonera could be due to the low sample sizes, which may have biased our estimations of blood parasite prevalence in these two populations (Jovani & Tella, 2006). Even so, results from the other populations suggest a complete absence of infection by blood parasites in nestlings. In a previous study, Gangoso *et al.* (2010) reported the absence of antibodies against the mosquito-borne West Nile virus in Eleonora's falcon nestlings from Alegranza, despite being detected in 14.8% of adults from the same population. This finding agrees with the results of our study regarding different vector-borne pathogens. In addition, Martínez-Abraín and Urios (2002) found no blood parasites infecting Eleonora's falcon nestlings from the Columbretes Islands. Nevertheless, Wink *et al.* (1979) found that 18.8% of adult Eleonora's falcons breeding in the Aegean Sea were infected by *Leucocytozoon*; regretfully, these authors provide no information about nestlings. Unlike our study, Martínez-Abraín and Urios (2002) and Wink *et al.* (1979) used blood smears for parasite detection. Nonetheless, our findings, in which a molecular approach was used, agree with the results of these authors' studies.

Although information regarding the development of blood parasites in nestlings of wild bird populations is scarce, studies conducted in different avian groups have detected avian blood parasites infecting nestlings after as few as 13 days of life (Martínez-de la Puente *et al.* 2013; Merino *et al.* 1995; Lobato *et al.* 2005). Svobodová *et al.* (2015) found *Haemoproteus* and *Leucocytozoon* in 16–23-days old sparrowhawk (*Accipiter nisus*) nestlings. Therefore, it is unlikely that the absence of parasites in Eleonora's falcon nestlings was due to time constraints in parasite development.

The presence of appropriate insect vectors is a crucial factor influencing the success of blood parasite transmission in birds (Sol *et al.* 2000; Martínez-de la Puente *et al.* 2013). Mosquitoes (Fam. Culicidae), biting midges (Fam. Ceratopogonidae) and black flies (Fam.

Simuliidae) are the main vectors of *Plasmodium*, *Haemoproteus* and *Leucocytozoon*, respectively (Valkiūnas, 2005). Although we did not perform any entomological surveillance to quantify insect diversity and abundance in the study areas, no previous study has ever found any of these insect vectors on either Alegranza (Gangoso *et al.* 2010) or the Columbretes Islands (Martínez-Abraín et. Al., 2002) Eleonora's falcons are usually parasitized by blood-sucking louse flies (Hippoboscidae) [Gangoso *et al*. 2010; Wink *et al.* 1979), which, in the absence of other vectors, could play a role in the transmission of blood parasites in marine habitats. Indeed, a recent study reported that *Haemoproteus iwa* in Galapagos great frigatebirds (*Fregata minor*) was vectored by a hippoboscid fly, an obligate ectoparasite of the bird host (Levin *et al.* 2012). However, louse flies can transmit parasites of the subgenus *Haemoproteus*, as is the case of *H. iwa* (Valkiūnas, 2005), but not of the subgenus *ParaHaemoproteus*, which were isolated from the adult Eleonora's falcons in this study. In addition, the subgenus *Haemoproteus* seems to be restricted to pigeons and frigatebirds (Martinsen *et al.* 2008; Merino *et al.* 2012) and, to our knowledge, have not been found to infect falcons. Therefore, the lack of suitable vectors might explain the incapacity of transmission from infected adults to uninfected nestlings in breeding areas.

Alternatively, the lack of blood parasites in Eleonora's falcon nestlings in these five populations could be due to host-related immune mechanisms, as suggested by Martínez-Abraín *et al.* (2004). However, this possibility is poorly supported by our results, since the prevalence of infection in adults found in this and previous studies (Wink *et al.* 1979) showed that Eleonora's falcons are indeed susceptible to blood parasite infections. The Eleonora's falcon possesses a very specialized Major Histocompatibility Complex (MHC), characterized by a complete lack of variability at both MHC class I and II, probably due to pathogen-driven selection (Gangoso *et al.* 2012) The MHC system may play a key role in the defence of birds against blood parasites (Westerdahl *et al.* 2005). Further studies should be conducted to identify the role of the specialized MHC system in the mechanisms used by Eleonora's falcon against blood parasite infections.

We suggest that either during migration or in wintering areas, adult Eleonora's falcons may encounter a diversity of vectors transmitting *Plasmodium*, *Haemoproteus* and *Leucocytozoon*. After breeding, Eleonora's falcons perform a long-distance migration

across continental Africa to reach Madagascar (Gschweng *et al.* 2008; Mellone *et al.* 2013), thus crossing and stopping in areas with a high abundance of potential insect vectors during the rainy season (Mellone *et al.* 2013; López-López *et al.* 2010). Njabo *et al.* (2011) and Waldenström *et al.* (2002) isolated *Plasmodium* and *Haemoproteus* parasites in wild mosquitoes from Cameroon and in migratory and resident birds from Nigeria, respectively. In addition, *Haemoproteus* (17.4% prevalence), *Leucocytozoon* (9.4%) and *Plasmodium* (1.9%) have been found in birds from different families in Madagascar (Savaje *et al*. 2009). In their wintering quarters, Eleonora's falcons inhabit degraded humid forests and cultivated areas close to pristine humid forest (Mellone *et al*. 2012a) where, due to their suitability for insect vector reproduction, blood parasite transmission may occur. Furthermore, during the pre-breeding and breeding seasons, adult Eleonora's falcons often travel inland (i.e. the main islands of the Canary and Balearic archipelagos, continental Africa and continental Europe) to visit freshwater ponds and other water bodies (Mellone *et al*. 2012b), where the presence of suitable vectors such as biting midges (Martínez-de la Puente *et al.* 2012a) and mosquitoes (Martínez-de la Puente *et al*. 2012b) has been recorded. By comparing the parasite lineages isolated from Eleonora's falcons with those previously found in other wild bird species, it is possible to infer areas of parasite transmission and determine the host-range of these parasite lineages. In this respect, *Plasmodium* LK6 and *Haemoproteus* LK4 lineages have been isolated from adults of the closely related lesser kestrel (*Falco naumannii*) in Spain (Ortego *et al*. 2007a,b), with a parasite prevalence of 4.6% and 0.7% in adults, respectively. Like Eleonora's falcon, the lesser kestrel is a long-distance migratory species that winters in Africa, which suggests that parasite-vector interactions in wintering quarters may facilitate the transmission of blood parasites in these species. In addition, the *Leucocytozoon* L_CIAE02 lineage was found in both adults and juveniles of the migratory black kite (*Milvus migrans*) in Tarifa (South Spain) (Pérez-Rodríguez *et al.* 2013), which suggests that this parasite lineage could be transmitted in both Africa and Europe. Interestingly, the two additional parasite lineages that we found in Eleonora's falcons have previously been isolated from non-raptor species. *Haemoproteus* hBUBIBI01, which only differs in a single nucleotide from the lineage LK4, has been isolated for the first time from cattle egrets (*Bubulcus ibis*) in southern Spain (Ferraguti *et al.* 2013b). Likewise, Illera *et al.* (2008) have reported the presence of *Plasmodium* TF413,

which is identical to lineage LK6, in Berthelot's pipits (*Anthus berthelotii*), a resident species presents in all the islands in the Canary archipelago. These latter authors (Illera *et al*. 2008) suggest that lesser kestrels, the only species previously found to be infected by the *Plasmodium* linage LK6, could have spread this lineage to Berthelot's pipits. However, lesser kestrels do not breed in the Macaronesian islands and only vagrant individuals are ever observed in this area. In this respect and according to our results, a long-distant migratory raptor such as Eleonora's falcon could spread blood parasites to resident birds on the main islands, where insect vectors are present (see Waldenström *et al*. 2002).

Conclusions

Our results support the hypothesis proposed by Piersma (1997) that explains the low prevalence of parasites in avian species living in marine environments and strongly indicates that in Eleonora's falcons habitat characteristics affect the transmission of blood parasites.

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Chapter 7

Genetic colour polymorphism is associated with avian malaria infections

Laura Gangoso, Rafael Gutiérrez-López, Josué Martínez-de la Puente, Jordi Figuerola. (*Biology letters, 2016; 12:20160839*)

Abstract

Individual genetic diversity is predicted to influence host–parasite interactions. Together with the genes directly associated with immune responses, variation in genes regulating vertebrate melanin-based pigmentation may play an important role in these interactions, mainly through the pleiotropic effects that affect colour- specific physiology, behaviour and immunity. Here, we test the hypothesis that the prevalence of avian malarial parasites differs between phenotypes in a raptor species in which the genetic basis of colour polymorphism and its pleiotropic effects over immune functions are known. We found that dark morphs had a higher prevalence of *Plasmodium* parasites than pale ones but detected no such association for *Haemoproteus*. This pattern may be associated with unequal exposure to vectors or, as suggested by our circumstantial evidence, to a differential ability to mount an immune response against blood parasites.

Introduction

Understanding the role of individual genetic diversity in resistance to infectious dis- eases is crucial for forecasting evolutionary responses and long-term conservation of host populations (Carius *et al*. 2001; Thompson *et al*. 2005). In birds, genetic colour polymorphism—defined as a highly heritable variation in expressed plumage coloration that is independent of age and sex—is often associated with variation in life-history traits, including physiology, behaviour and immunity (Ducrest *et al*. 2008; Roulin & Ducrest, 2011). These associations may result from pleiotropic effects of genes regulating melanogenesis, such as the melanocortin-1-receptor (*Mc1r*). For example, pharmacological research has shown that melanocortin receptors and their ligands are key regulators of immune functions. *Mc1r* is constitutively expressed on monocytes/macrophages, but also on dendritic cells and lymphocytes with anti- gen-presenting and cytotoxic functions. The activation and binding of the peptide alpha-melanocyte-stimulating hormone (a-MSH) to its receptor MC1R in non- melanocytic immune cells modulates both the innate and the acquired immune responses, with overall anti-inflammatory and, apparently, immunosuppressive effects (Gangoso *et al*. 2015). On the other hand, it has been proposed that the phagocytic function of melanocytes could confer higher protection from pathogens to more melanized individuals (Chakarov *et al*. 2008).

Parasites of the genera *Plasmodium*, *Haemoproteus* and *Leucocytozoon* are all pathogenic to some degree, yet *Plasmodium* is considered as the most virulent one (van Riper *et al*. 1994). Parasite lineages exhibit antigenic differences that will influence the effectiveness of the bird immune system. Consequently, virulence strongly depends on the interplay between specific lineages and the ability of the avian host to cope with the parasite infection (Bensch *et al*. 2007). In birds that survive infection, the initial acute phase, when severest fitness consequences generally occur, is followed by a rapid decline in parasitaemia to chronic levels with lower fitness consequences for the bearer (van Riper *et al*. 1994; Bensch *et al*. 2007). Immune response to malarial infection is mainly cell-mediated through the lymphoid-macrophage system, while anti- bodies play an important supportive role (Bensch *et al*. 2007). Although the precise mechanism is unclear, a number of studies have proposed that the adaptive function of melanin-based colour polymorphism

is associa- ted with parasite resistance and could cause differences in vector-borne parasite loads between morphs (e.g. (Galeotti & Sacchi, 2003; Jacquin *et al*. 2011).

Eleonora's falcon (*Falco eleonorae*) is a migratory raptor that breeds throughout the Mediterranean basin and winters in Madagascar. It occurs in two distinct melanin-based colour morphs owing to variation in the *Mc1r* gene (Gangoso *et al*. 2011). Although the relationship between coloration and blood parasite infection in this species is unknown, both inflammatory and humoral immune responses are lower in dark than in pale nestlings (Gangoso *et al*. 2011, 2015). Therefore, in the light of the link between Mc1r-genotypes and both arms of the immune system, we hypothesize that the two morphs will differ in parasite prevalence because dark morphs are less able to cope with parasite infections (genetic link hypothesis). Alternatively, parasite prevalence could differ due to morph-specific exposure to vectors, either if both morphs exploit different habitats with different vector abundances or if both morphs are differently appealing to vectors, thus creating unequal infection probabilities (exposure hypothesis).

Material and methods

Sampling was conducted in July – October in Alegranza islet (Canary Islands; 1050 ha, 289 m above sea level). Adult Eleonora's falcons were captured every year (mean 1⁄4 23.22 individuals, range 1⁄4 5 – 47), and their colour morph was determined visually (Gangoso *et al*. 2011). All birds were weighed and their wing length measured. Blood samples were preserved in absolute ethanol and stored at 2208C until molecular analysis was performed. All birds were marked with numbered rings and released after manipulation.

DNA extraction and blood parasite determinations

We analysed 209 blood samples from 183 individuals: 151 pale morphs (91 females and 60 males) and 32 dark morphs (22 females and 10 males). The remaining 26 samples belonged to 19 individuals recaptured in successive years. Genomic DNA was used to determine the prevalence of *Plasmodium*, *Haemoproteus* and *Leucocytozoon* parasites following (Hellgren *et al*. 2004) (see the electronic supplementary material).

Statistical analyses

The probability of different morphs being infected by blood parasites was assessed using generalized linear mixed models (GLMMs) with binomial error and logit link function in R v. 3.0.2 (R Core Team, 2015) using the dataset available in (Gangoso *et al*. 2016). To prevent pseudo- replication, we used a random subsampling (1000 iterations) of the 19 resampled birds for each parasite genus (see the electronic supplementary material; https://figshare.com/articles/Extended_methods_from_Genetic_colour_polymorphism_is_associated_with_avian_malarial_infections/4308098). The infection by *Plasmodium* and *Haemoproteus*, respectively, was defined as a binary variable (0/1) and used as the response variable. The morph type, sex (only for *Plasmodium*) and their interaction were included as fixed factors. We also included a body-condition index as a covariate, estimated for each sex separately as the standardized residuals of a linear regression of body mass on wing length. Year was included as a random term. We did not perform a third model for *Leucocytozoon*, because only one individual was found to be infected by this para- site. Parameter estimates and standard errors from the resulting GLMMs were averaged and the range of p-values and percentage of models where each term was statistically significant calculated.

Results

Overall, the prevalence of blood parasites was 12.9% (figure 1; electronic supplementary material, table S1 for details on parasite lineages, Gangoso *et al*. 2016). Of the 19 resampled individuals, 13 (10 pale females and three dark females) were never infected. However, one and two pale females became infected by *Plasmodium* and *Haemoproteus*, respectively, between the first and second sampling period. By contrast, three pale females, two infected by *Haemoproteus* and one by *Plasmodium*, were found to be uninfected 1 year later.

Dark falcons had a greater *Plasmodium* prevalence than pale ones (mean estimate = 2.50+0.01 s.e., p-range = 0.001–0.01; figure 1). Sex was not significant (mean estimate = 0.75+ 0.01 s.e., p-range = 0.59 – 0.99), whereas body condition (mean estimate = 0.011 + 0.001 s.e., p-range = 0.02 – 0.59) and the interaction between sex and morph (mean estimate = 22.20 + 0.012 s.e., p-range = 0.03–0.22) were significant only in 6.5% and 16.6% of the models, respectively. There was no significant relationship between the

probability of infection by *Haemoproteus* and any explanatory variable (in all cases p-range = 0.30–0.99).

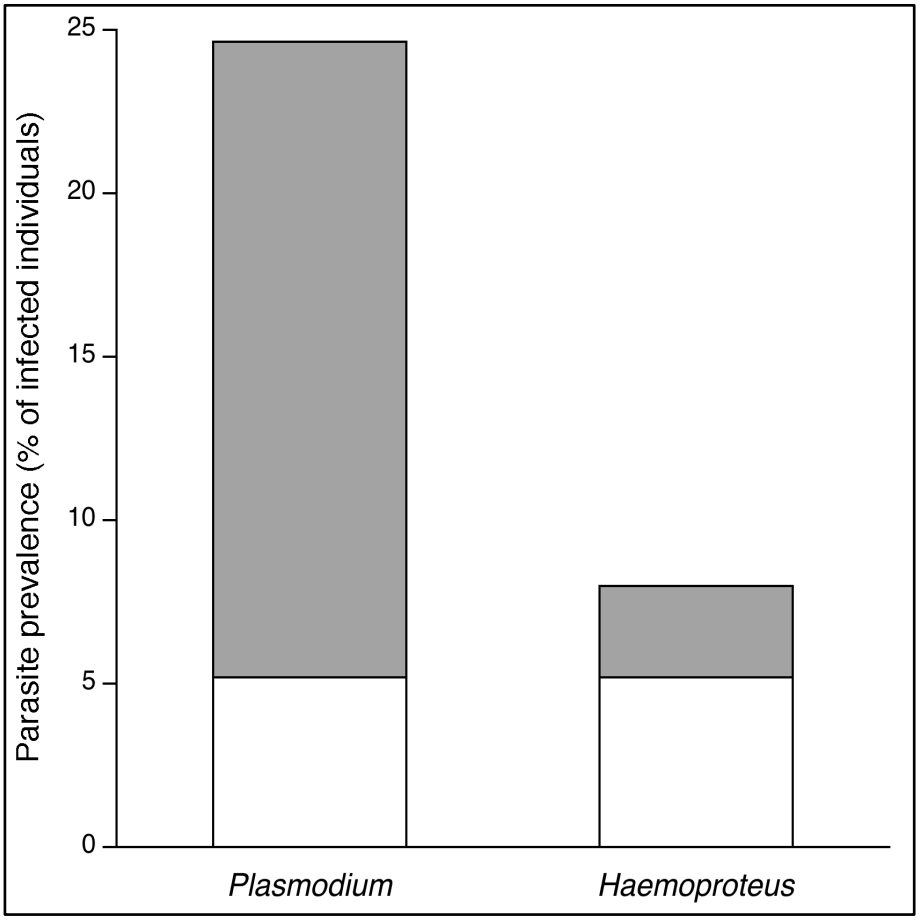

Figure 1. Prevalence (number of infected/total 100) of the two blood parasite genera infecting adult Eleonora's falcons of pale (white) and dark (grey) morph.

Discussion

We found that dark falcons had a higher prevalence of *Plasmodium*, the commonest parasite genus, than pale ones but found no significant relationship for *Haemoproteus*. Different factors such as differential exposure to vectors, the differing virulence of parasite genus/lineages and/or the host's capacity to fight infections may influence this result. In the support of the first possibility, Galeotti & Sacchi (2003) found that rufous-morph tawny owls (*Strix aluco*) hosted higher total blood parasite burdens than grey morphs owing to

both greater exposure to vectors and greater susceptibility to parasites. In feral pigeons (*Columba livia*), alternative morphs were distributed non-ran- domly across an urban gradient and had different parasite risks (Jacquin *et al*. 2013). However, the different Eleonora's falcon morphs inhabit small islands sympatrically during the breeding season and local transmission of blood parasites at breeding grounds is absent, owing to the lack of suitable vectors (Gutiérrez-López *et al*. 2015). Therefore, differences in the exposure to vectors must occur during migration and/or in their wintering quarters, where insect vectors abound (Tantely *et al*. 2016) and the parasite transmission is likely to be higher.

The most prevalent *Plasmodium* LK6 is thought to be trans- mitted by *Culex pipiens*, while P_ACCTAC01 is transmitted by *Coquillettidia aurites* (see the electronic supplementary material, table S1), and these are common mosquitoes in Africa and Madagascar (Tantely *et al*. 2016). The lineage LK6 was recently iso- lated from passerines from Macaronesian archipelagoes, the Iberian Peninsula and Morocco (electronic supplementary material, table S1). Although the transmission areas remain unclear, it has been proposed that migratory birds such as Eleonora's falcon could spread blood parasites to resident birds on the main islands, where insect vectors are present (Gutiérrez-López *et al*. 2015). It has been suggested that darker colours are more attractive to mosquitoes than light colours and so entirely dark plumages could increase host–vector contact rates. However, feeding preferences of these mosquitoes with regard to colour attractive- ness are unknown. In addition, data on GPS-tagged falcons do not indicate the existence of morph-specific habitat exploitation during winter (L Gangoso, J Figuerola 2015, unpublished data).

This suggests that the difference in prevalence between morphs is unlikely to be due to morphspecific exposure to vectors but probably results from differential abilities to mount an immune response. Pale falcons could be more susceptible to *Plasmodium* infection than dark ones and their lower prevalence could in turn be the reflection of greater mortality. No study has addressed the effects of LK6 on host survival (electronic supplementary material, table S1; https://figshare.com/articles/Parasite_lineages_from_Genetic_colour_polymorphism_is_associated_with_avian_malarial_infections/4308092). However, we cannot rule out a selective disappearance of pale morphs due to a higher mortality during the acute phase of

infection. Dark Eleonora's falcons have poorer immune responses than pale ones from the nestling stage onwards (Gangoso *et al*. 2011, 2015). It is thus likely that dark falcons have lower immune capacities in adulthood since this negative relationship is due to their *Mc1r* derived genotype and not to the environment (Gangoso *et al*. 2015). The fact that three infections found in pale females became undetectable in successive years partially supports the idea of greater immune competence in pale falcons for fighting infections. Nonetheless, the effects of infection can greatly depend on the parasite load. Previous studies addressing the relationship between plumage coloration and blood parasites have found differences in infection intensity rather than in prevalence, thereby suggesting that differences are due to resistance to parasites rather than exposure to vectors (Chakarov *et al*. 2008; Jacquin *et al*. 2011; Lei *et al*. 2013). However, we estimated prevalence rather than infection intensity because birds were caught during a relatively long period (from arrival at breeding grounds to fledglings' emancipation) and across years. Infection intensity may vary greatly along and between breeding seasons (Merino *et al*. 2004), thus making between-individuals comparisons difficult to interpret. Further experimental approaches would be needed to clarify the relationship among colour polymorphism, blood parasite intensity and immune competence.

In conclusion, our results are in accordance with the genetic link hypothesis, yet we cannot completely rule out the exposure hypothesis and both mechanisms could contribute to the skewed prevalence of *Plasmodium* to the dark morph. To the best of our knowledge, this is the first study addressing the relationship between colour polymorphism and parasite prevalence in which both the gene responsible for colour polymorphism and its pleiotropic effects on immune functions are known, which thus enabled us to infer potential mechanisms underlying this covariation.

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Chapter 8

Louse flies of Eleonora's falcons also feed on their prey: an alternative transmission pathway for blood parasites or an evolutionary "cul de sac"?

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Abstract

Host shifts are widespread among avian Haemosporidians. Nonetheless, the success of transmission to a new host depends on the parasite-host and parasite-vector compatibility. Insular avifaunas are characterized by the low prevalence and diversity of Haemosporidians, although the underlying ecological and evolutionary processes remain unclear. Here, we investigated the role of louse flies in the transmission networks of Haemosporidians in an insular ecosystem. Owing to the low diversity of parasites previously found infecting Eleonora's falcons and the presence of only one potential vector on the island, we expected to find a limited host-vector-parasite system. However, we found an apparent transmission failure of great diversity of parasite lineages. Phylogenetic analyses showed that the 23 lineages found in louse flies spread across the existent *Haemoproteus* and *Plasmodium* clades, all of them typical of Passeriformes. Seven of 18 parasite lineages isolated from bird preys were not found in louse flies. We found molecular evidence that louse flies also feed on passerines hunted by Eleonora's falcons and the commonest parasites of louse flies were found in the more frequent prey species. The lack of infection in nestlings and the mismatch between the lineages isolated here and those previously found in adult falcons suggest that despite louse flies contact with a diverse array of parasites, no successful transmission to Eleonora's falcon occurs. This could be due to the falcons' resistance to infection, the inability of parasites to develop in these phylogenetically distant species and/or the inability of Haemosporidian lineages to complete their development in louse flies.

Introduction

Hosts and parasites are engaged in a pervasive evolutionary arms race to maximize own fitness (Thompson, 1998). At ecological time scales, parasites tend to be locally and temporally adapted to their hosts, but host shifts are widespread across parasite taxa (e.g. fungi (McTaggart *et al.* 2016), bacteria (Delaney *et al.* 2012) and viruses (Longdon *et al.* 2014)). Host shifts have the potential for rapid parasite diversification and also for causing emerging infectious diseases (Woolhouse *et al.* 2005). This becomes more complicated when parasites require a vector to be transmitted between vertebrate hosts, such as protozoan blood parasites, yet host switchings are also frequently observed (Escalante *et al.* 1998; Fecchio *et al.* 2018; Ricklefs & Fallon, 2002; Ricklefs *et al.* 2004; Waldenström *et al.* 2002). Nonetheless, factors determining the success or failure of these events are complex and strongly dependent on ecological (e.g. climate, geographic or vector-imposed barriers) and finely tuned parasite/host-related processes, such as host specificity and adaptability of the parasite as well as host immune mechanisms (Gager *et al.* 2008; Lee *et al.* 2017; Moens *et al.* 2016; Sieber & Gudelj, 2014).

The biting behavior of blood-feeding arthropods can largely determine the host-parasite contact rates and hence, the transmission networks of vector-borne parasites (Martínez-de la Puente *et al.* 2015; Takken & Verhulst, 2013; Yan et al. 2017). Blood parasites infecting a particular host may interact with a diversity of blood feeding arthropods that are competent or refractory for the transmission of the pathogen. Avian malaria parasites of the genera *Plasmodium* and the phylogenetically related *Haemoproteus*, although having similar life cycles, are transmitted by different dipterian insect vectors. While mosquitoes (Culicidae) transmit *Plasmodium*, *Culicoides* (Ceratopogonidae) and louse flies (Hippoboscidae) are the main vectors of *Haemoproteus* parasites of the sub-genera *Parahaemoproteus* and *Haemoproteus*, respectively (Valkiūnas, 2005). However, insect vectors of these avian parasites seem to have a relatively opportunistic behavior, feeding on blood of different bird species potentially leading to host-switches. Indeed, host shifts are frequent and fast processes that have actually shaped the evolutionary history of avian Haemosporidians (Alcala *et al.* 2017; Ricklefs *et al.* 2014). Bird-parasite interactions have been intensively studied to identify the specificity among avian Haemosporidians and different hosts (Clark *et al.* 2014;

Križanauskienė *et al.* 2006; Palinauskas *et al*. 2008; Valkiūnas, 2005). However, very little attention has been paid to the occurrence of vector shifts and, in general, to the role of the feeding behavior of vectors in enabling or hampering host shifts (Gager *et al.* 2008; Ishtiaq *et al.* 2008; Kim & Tsuda, 2010). This is partially due to the fact that vector breadth of the astounding diversity of within-genera avian malaria lineages is poorly known (Atkinson & van Riper, 1991; Njabo *et al.* 2011; Valkiūnas, 2005).

Environmental conditions and their effects on vector populations strongly affect the transmission dynamics of avian Haemosporidians (Ferraguti *et al.* 2018). On oceanic islands, where populations of insect vectors are usually limited by the prevailing conditions of high wind speeds and salinity, louse flies may play a key role in haematozoan transmission. For example, frigate birds are commonly infected by *Haemoproteus* parasites, including *Haemoproteus* (*Haemoproteus*) *iwa*, which are vectored by louse flies (Levin *et al.* 2011; Merino *et al.* 2012). Likewise, *Haemoproteus* (*Haemoproteus*) multipigmentatus infecting endemic Galápagos doves (Zenaida galapagoensis) is transmitted by the louse fly Microlynchia galapagoensis (Valkiūnas *et al*. 2010). In an insular ecosystem, the opportunities for parasite spillover and diversification can be limited owing to the low number of interacting species and diversity of habitats. On the other hand, vacant niches in the form of new vectors and hosts become available for new arriving parasites, either for generalist strategists capable of exploiting new opportunities and for parasites able to co-evolve in new host-vector assemblages, thus broadening host ranges or promoting parasite diversification, respectively (Agosta & Klemens, 2008; Drovetski *et al.* 2014; Medeiros *et al*. 2014; Santiago-Alarcon *et al*. 2014). In this context, it is known that louse flies are able to move between host individuals of the same or even different species, potentially increasing the probability of host switching by Haemosporidians (Jaramillo, Rohrer, & Parker, 2017; Levin & Parker, 2014). This may be the case of *H. multipigmentatus*, for which louse flies could have allowed parasites to jump from doves to distantly related avian hosts on oceanic islands (Jaramillo *et al.* 2017; Levin *et al.* 2011; Levin & Parker, 2014).

In this study, we investigated the role of louse flies in the transmission networks of avian Haemosporidians in an insular ecosystem. The Eleonora's falcon (*Falco eleonorae*) is a medium-sized long-distance migratory raptor that breeds on islands over the

Mediterranean basin and winters in Madagascar (Kassara *et al.* 2017; Walter, 1979). During the breeding season, Eleonora's falcons feed on European migratory birds intercepted over the ocean while heading to Africa, and hunted prey are stored in larders around the nest sites (Viana *et al*. 2016). Adult Eleonora's falcons are commonly infected by *Plasmodium* and *Haemoproteus* parasites (Gangoso *et al.* 2016) while nestlings are uninfected (Gutiérrez-López *et al.* 2015a), suggesting the lack of transmission at breeding areas. In addition, both adult and nestling Eleonora's falcons are heavily parasitized by the louse fly Ornithophila gestroi (Gangoso *et al.* 2010), potentially affecting the transmission dynamics of blood parasites at breeding grounds, as in the case of different *Haemoproteus* lineages in marine ecosystems (Levin *et al.* 2011; Levin *et al.* 2012; Valkiūnas *et al.* 2010). Ornithophila gestroi had been reported only on Eleonora's falcon and the closely related Common and Lesser kestrels (*Falco tinnunculus* and *F. naumanni*) (Beaucournu *et al*. 1985; Gangoso *et al.* 2010; Walter, 1979). Consequently, we hypothesize that Eleonora's falcons and *O. gestroi* louse flies will share infection by the reduced number of haemosporidian lineages reported in the Eleonora's falcon.

Methods

We sampled louse flies in September 2011–2013 on Alegranza islet (Canary Islands; 1050 ha, 289 m.a.s.l.). Nestling Eleonora's falcons of 20-25 days-old were inspected for louse flies during 5-min, focusing on the area peri-cloacal, where these insects usually concentrate (authors' pers. ob.). Louse flies (range 1-9) were removed from each individual bird and immediately introduced in absolute ethanol (2011; 159 louse flies sampled from 50 nests) or kept alive during four days in empty plastic recipients before introducing them in Eppendorf tubes filled with absolute ethanol (2012 and 2013, corresponding to 369 louse flies from 64 nests and 499 louse flies from 63 nests, respectively). This later procedure allowed the digestion of a blood meal potentially present in the insect abdomen. Host DNA is usually degraded after few days post ingestion (Martínez-de la Puente *et al*. 2013 and references therein). A single louse fly species has been collected in the study area corresponding to *O. gestroi*, according to morphological and genetic characterization of specimens (Gutiérrez-López et al. 2015b).

To identify the bird hosts of blood parasites potentially isolated from louse flies, blood samples from both adult and nestling Eleonora's falcons and bird preys were obtained. The blood parasites infecting Eleonora´s falcons sampled from 2006 to 2014 were previously analyzed (Gangoso *et al.* 2016; Gutiérrez-López *et al.* 2015a). In addition, in September 2013 we sampled 90 fresh preys of 12 bird species belonging to 7 different families. We obtained a fresh blood sample (N= 14) or heart tissue with nearly coagulated blood (N = 76) from each bird that were immediately stored in Eppendorf tubes filled with absolute ethanol. We left the sampled prey in the same place where found for later consumption by the falcons. All samples were preserved at -20ºC until molecular analysis was performed.

Molecular analyses

Was extracted genomic DNA from whole louse flies collected in 2011 using a common chloroform/isoamyl alcohol protocol and DNA from the head-thorax of louse flies collected in 2012-13 with the MAXWELL® 16 LEV Blood DNA Kit (see Gutiérrez-López *et al.* 2015b). For the louse flies collected in 2012-13, the head-thorax of each fly was separated from the abdomen using sterile scalpel blades and forceps on sterile Petri dishes. The abdomens were kept in individual tubes with absolute ethanol. We isolated genomic DNA from blood samples or heart tissue from fresh bird preys of Eleonora´s falcons by using the Maxwell-based protocol. Fresh organs including heart can be successfully used to identify exoerythrocytic stages of avian malaria infections using molecular tools, although the prevalence of infection could be underestimated (Mendes *et al.* 2013).

We determined the presence and identity of *Haemoproteus* and *Plasmodium* DNA in head-thorax of louse flies and bird preys following Hellgren *et al*. (2004). The presence of amplicons was verified in 1.8% agarose gels and positive samples were sequenced using the BigDye technology (Applied Biosystems) or the Macrogen sequencing service (Macrogen Inc., Amsterdam, The Netherlands). Sequences were edited using the software Sequencher™ v 4.9 (Gene Codes Corp., © 1991–2009, Ann Arbor, MI 48108) and assigned to parasite lineages/morphospecies after comparison with GenBank (National Centre for Biotechnology Information) and MalAvi (Bensch, Hellgren, & Pérez-Tris, 2009)

databases. Sequences identified for the first time in this study were deposited in the GenBank database.

The abdomen of 80 louse flies with a positive amplification of parasites (see below) in the head-thorax was further analyzed to discriminate if the parasite identified could be due to any rest of an undigested blood meal. To do that, we extracted genomic DNA using the Maxwell-based protocol described above and determined their blood parasite infection status following Hellgren et al. (2004). For those abdomens (n=18) showing positive amplifications of parasite DNA, we performed a nested PCR to identify the host species of any potential remain of a previous blood meal (Alcaide *et al.* 2009).

Statistical and phylogenetic analyses

We assessed differences in prevalence in louse flies across years by means of contingency tables in JMP software (SAS Institute, Cary, NC). The similarity and diversity of lineages sequenced in the louse flies and the migratory birds were compared with the Jaccard similarity index (Jaccard, 1902), which ranges from 0 (no similarity) to 1 (complete similarity). For these calculations, we removed a single *Haemoproteus* sequenced from a louse fly in 2011 because it was not possible to identify the precise lineage due to the low quality of the sequence. The statistical significance of the result was established using the critical value of Jaccard's similarity index at the 95% confidence level (Real, 1999).

We assessed the phylogenetic relationships of the 21 *Haemoproteus* and 10 *Plasmodium* lineages found here with sequences from 69 *Haemoproteus* and 29 *Plasmodium* lineages of known morphospecies deposited in MalAvi (Bensch *et al.* 2009; accessed May 2018). Sequences were aligned using the CLUSTALW algorithm implemented in MEGA7 (Kumar *et al*. 2016). We used 478 pb fragments to analyze the phylogenetic relationships between lineages using the maximum likelihood algorithm based on the Jukes-Cantor model (Jukes & Cantor, 1969). Nodal support was estimated by bootstrap analysis with 1000 replicates (Felsenstein, 1981). We used one sequence of *Leucocytozoon* corresponding to the lineage L_CIAE02 as out-group.

Results

Haemosporidian parasites were isolated from 28 % of the analyzed louse flies (N = 1027). Infected flies were found in 32, 42 and 39 different nests in 2011, 2012 and 2013, respectively. Parasite prevalence was significantly higher in 2011 (40.25%) than in 2012 (24.12%) and 2013 (26.85%) (Pearson $\chi2 = 14.94$, df = 2, p = 0.0006) but did not differ between these later two years (Pearson $\chi2 = 0.83$, df = 1, p = 0.36). It is important to note that the higher prevalence found in 2011 could result from the different methodology used this year (see methods). The prevalence of *Haemoproteus* was much higher than that of *Plasmodium* in all study years, accounting for 259 and 18 of total infections, respectively (Pearson $\chi2 = 6.26$, df = 1, p = 0.01). The prevalence of *Haemoproteus* differed across years (Pearson $\chi2 = 13.45$, df = 2, p = 0.001), with the highest prevalence found in 2011 (36.60 %) and the lowest in 2012 (21.25 %). Nonetheless, prevalence of *Haemoproteus* did not differ between 2012 and 2013 (Pearson $\chi2 = 1.78$, df = 1, p = 0.18). The prevalence of *Plasmodium* did not differ over time (Pearson $\chi2 = 1.55$, df = 2, p = 0.46). Ten louse flies showed evidence of coinfection by *Haemoproteus* and *Plasmodium* based on the double peaks found in the chromatograms and were not included in previous statistical tests.

Overall, 24 different parasite lineages were identified in louse flies (18 *Haemoproteus* spp. and 6 *Plasmodium* spp., Table 1). One amplification was only identified to the genus level, corresponding to a *Haemoproteus* parasite. The commonest parasite lineage isolated from louse flies was *H.* PFC1 (48.8% of total infections, N= 287), followed by *H.* HIPOL1 (24.7%). The remaining parasites were found in ≤ 7 louse flies (see Table 1). Sixteen of the parasite lineages isolated from louse flies were previously found infecting avian species (see Table S1) and two new lineages were also shared with bird prey (see below), while other five isolated only from louse flies are described here for the first time (lineages named as: ORGES1, ORGES2, ORGES3, ORGES4 and ORGES5, GenBank accession numbers: MH271176-80).

Table 1. Identified *Haemoproteus* and *Plasmodium* lineages found in louse flies and bird preys in this study. The new lineages isolated are indicated with an *.

	Louse flies				Bird prey
Lineages	2011	2012	2013	Total	2013
H. sp. LANSEN1*	0	0	3	3	5
H. sp. ACDUM2	0	1	1	2	3
H. sp. HIPOL4*	1	2	5	8	2
H. attenuatus ROBIN1	1	0	0	1	3
H. balmorali COLL3	1	3	1	5	2
H. balmorali SFC1	0	2	0	2	
H. sp. ERU-15H	1	1	7	9	
H. sp. HIPOL1	20	15	36	71	12
H. sp. ORGES1*	0	1	0	1	
H. sp. ORGES2*	0	1	0	1	
H. sp. ORGES3*	0	0	1	1	
H. sp. ORGES4*	0	0	1	1	
H. sp. ORGES5*	1	0	1	2	
H. pallidus PFC1	27	50	63	140	19
H. palloris WW1	0	2	4	6	
H. payevsyi RW1	0	0	1	1	
H. sp. RBS3	2	0	1	3	1
H. (unidentified lineage)	2	0	0	2	
H. sp. HIPOL5*					2
H. sp. PHYBON1*					1
H. sp. PHYTRO1*					1
H. sp. SYCAN02*					1
P. sp. AFTRU5	1	1	0	2	
P. sp. LK6	0	2	0	2	
P. relictum GRW11	0	1	1	2	1
P. relictum SGS1	1	2	4	7	6
P. sp. SYAT24	0	0	1	1	
P. vaughani SYAT05	0	3	1	4	1
P. sp. COLL1					1
P. sp. GRW9					1
P. sp. MOALB1					1

Of the 80 louse flies collected in 2012 with infected head-thoraxes, 18 abdomens (22.5%) showed positive amplifications of parasites corresponding to the *Haemoproteus* lineages PFC1 (11.3%, N=9), HIPOL1 (3.8%, N=3), WW1 (2.5%, N=2), SFC1 (1.3%, N=1) and LK4 (1.3%, N=1), and the *Plasmodium* lineage LK6 (2.5%, N=2). Seven louse flies showed different parasite lineages in the abdomens than in the head-thorax. From these 18 positive abdomens, DNA from birds of the Falco genus and *Hippolais polyglotta* was

amplified from 11 and three louse flies, respectively. The blood meals of the remaining four louse flies were not possible to identify probably due to the high degradation of host DNA.

Overall, 74.44% (N = 90) of Eleonora´s falcon preys were infected by parasites corresponding to 6 lineages of *Plasmodium* spp. and 12 lineages of *Haemoproteus* spp, including 6 newly described lineages, two of which also shared with louse flies (i.e. LANSEN1 (MH271174) and HIPOL4 (MH271173), see Table 1). Blood parasites were isolated from all 12 bird species but two, i.e. *Coturnix coturnix* (N=2) and *Phoenicurus phoenicurus* (N=1) (Table 2). The bird species recorded more frequently as preys across years were the European pied flycatcher (*Ficedula hypoleuca*) and the Common whitethroat (*Sylvia communis*), while the frequency of other species differed over time (Table 2). European pied flycatchers were recorded in all nests where positive louse flies were found, while Common whitethroats were found in 35 out 38 nests. The most prevalent parasite lineages isolated from passerines were the same commonest lineages found in louse flies, i.e. *H.* PFC1 (28.4% of total infections, N= 67) and *H.* HIPOL1 (17.9%). The remaining lineages were found in ≤ 6 birds (see Table 1). Passerines had seven parasite lineages that were not found in louse flies, including the *Plasmodium* lineages COLL1, GRW9 and MOALB1 and the newly described *Haemoproteus* lineages HIPOL5 (GenBank reference: MH271175), *H.* PHYBON1 (MH271181), *H.* PHYTRO1 (MH271182) and *H.* SYCAN02 (MH271183).

Parasite faunas isolated in bird preys and louse flies showed moderate but not significant similarity, both when considering only parasite lineages isolated from louse flies collected in 2013, when preys were sampled (Jaccard coefficient = 0.40), and when combining parasites isolated in louse flies from all years (Jaccard coefficient = 0.37).

According to the phylogenetic analyses, parasites found in louse flies in this study showed close relationship with those previously isolated from Passeriformes (Fig, 1; see Table S1). The new lineages found in louse flies were distributed in different clusters including phylogenetically related lineages of known morphospecies: ORGES2 and ORGES5 were closely related to a lineage of *H. pallidus*, while *H.* ORGES3, *H.* ORGES4 and *H.* LANSEN1 (also isolated from bird prey) were closely related to *H. lanii*. The lineage *H.* ORGES1 was closely related to lineage *H.* SYCAN02, which was isolated from a bird prey

(*Sylvia cantillans*). The lineage *H*. HIPOL4 isolated from louse flies and birds was closely related to the morphospecies *H. belopolsky*. Of the lineages isolated only in bird preys, *H*. HIPOL5 was closely related to the morphospecies *H. palloris*, while the lineages *H*. PHYBON1 and *H*. PHYTRO1 clustered with lineages of *H. killagoi* and *H. majoris*, respectively.

Table 2. Bird species hunted by Eleonora's falcons during the study period and their relative frequency in 2013. The number of nests sampled each year is indicated below. The number of individuals of each species sampled in this study and the prevalence within each species of the different parasite lineages isolated (next to each parasite lineage, in parenthesis) are also shown.

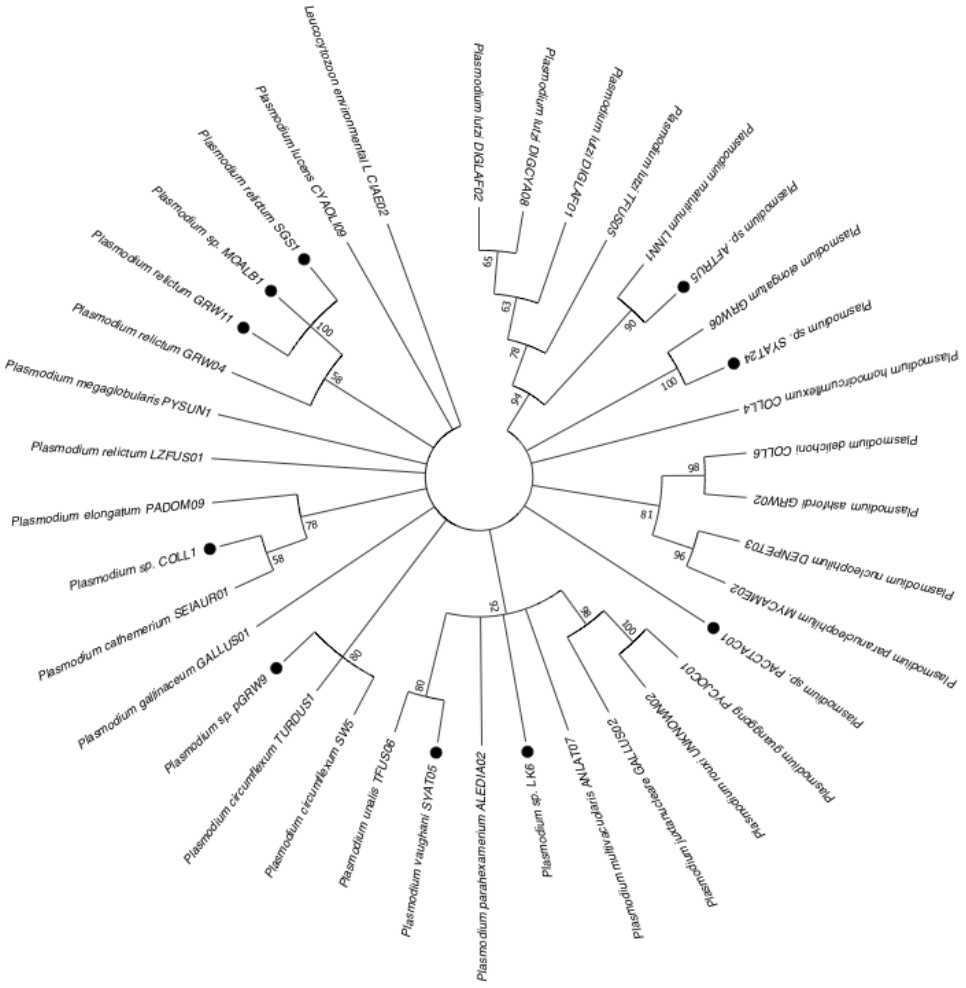
Bird species	2011 N=57	2012 N=43	2013 N=99	Frequency 2013	N sampled	Parasite lineages and prevalence within each bird species
Acrocephalus paludicola			2	0.002	2	*H. pallidus* PFC1 (0.5), *P. sp.* COLL1 (0.5)
Acrocephalus scirpaceus	2			0		
Actitis hypoleucos	7	4	44	0.05		
Anthus trivialis	1	1		0		
Apus sp.	1		18	0.02		
Bulweria bulwerii	1		32	0.04		
Cercotrichas galactotes	1			0		
Clamator glandarius			1	0.001		
Coturnix coturnix	10	12	34	0.04	2	0
Crex crex			7	0.008		
Cuculus canorus	3		9	0.01		
Ficedula hypoleuca	73	118	177	0.21	27	*H. pallidus* PFC1 (0.48), *H. sp.* LANSEN1 (0.04), *H. balmorali* COLL3 (0.07), *H. sp.* HIPOL1 (0.07), *P. sp.* GRW9 (0.04)
Hippolais polyglotta	33	44	59	0.07		
Hippolais sp.		3		0		
Hydrobates pelagicus	2		14	0.02		
Jynx torquilla	2	5	14	0.02		
Lanius senator	22	14	67	0.08	9	*H. sp.* LANSEN1 (0.44), *H. attenuatus* ROBIN1 (0.11), *H. sp.* RBS3 (0.11), *P. relictum* SGS1 (0.11)
Locustella naevia	21	3	7	0.008		
Luscinia megarhynchos	60	7	49	0.06	2	*H. attenuatus* ROBIN1 (1)
Motacilla flava		1	8	0.009	1	*P. sp.* MOALB1 (1)
Muscicapa striata	8	3		0		
Oenanthe hispanica			5	0.006		
Oenanthe oenanthe			38	0.05		
Phalaropus fulicarius		2	4	0.005		
Phoenicurus ochruros			34	0.04		
Phoenicurus phoenicurus	6	4	3	0.004	1	0
Phylloscopus bonelli	4	4	3	0.004	5	*H. sp.* HIPOL5 (0.2), *H. sp.* PHYBON1 (0.2), *P. relictum* SGS1 (0.2)
Phylloscopus collybita	8		4	0.005		
Phylloscopus sp.	13	11	39	0.05		
Phylloscopus trochilus	5	4	3	0.004	2	*H. pallidus* PFC1 (0.5), *H. sp.* PHYTRO1 (0.5)
Streptopelia turtur	2		12	0.01		
Saxicola rubetra		1		0		
Sylvia cantillans			2	0.002	2	*H. pallidus* PFC1 (0.5), *H. sp.* SYCAN02 (0.5)
Sylvia communis	70	47	139	0.17	21	*H. pallidus* PFC1 (0.14), *H. sp.* ACDUM2 (0.05), *H. sp.* HIPOL1 (0.09), *P. relictum* GRW11 (0.05), *P. relictum* SGS1 (0.2), *P. vaughani* SYAT05
Sylvia sp.	1	1		0		
Upupa epops	3	3	1	0.001		

Discussion

The prevalence and diversity of haemosporidian lineages in birds from islands are usually lower than that found in the mainland (Hellgren *et al.* 2011; Padilla *et al*. 2017; Pérez-Rodríguez *et al*. 2013; Sari *et al.* 2013). It has been proposed that the establishment of haemosporidian parasites into new areas strongly depends on vector distribution and vector switches (Ricklefs *et al.* 2002, 2004). However, the ecological and evolutionary processes underlying the colonization of new host-vector insular networks by blood parasites remain largely unknown. On the basis of the low prevalence and diversity of hemosporidian parasites infecting Eleonora's falcons (Gangoso *et al.* 2016; Gutiérrez-López *et al.* 2015a) and the presence of only one potential vector species on the island, we expected to find an essentially simple host-vector-parasite system.

However, we faced a rather complex scenario of apparent transmission failure of a great diversity of avian Haemosporidians. The unexpected high diversity of *Haemoproteus* and *Plasmodium* lineages found in louse flies were probably the result of the not so specialized feeding behavior of *O. gestroi*. We found a high prevalence and moderate similarity between the parasite faunas of passerine bird prey and louse flies. However, the lineages isolated in louse flies did not match with those previously found infecting Eleonora's falcons, with the exception of *Plasmodium* LK6. However, louse flies are not competent vectors for *Plasmodium* and consequently, Eleonora's falcon infections by this parasite should be the result of contacts with infected mosquitoes outside the breeding quarters (see Gangoso *et al.* 2016).

A)


Leucocytozoon environmental L C/AE02
Plasmodium lucens CYAOL09
Plasmodium relictum SGS1
Plasmodium sp. MOALB1
Plasmodium relictum GRW11
Plasmodium relictum GRW04
Plasmodium megaglobularis PYSUN1
Plasmodium relictum LZFUS01
Plasmodium elongatum PADOM09
Plasmodium sp. COLL1
Plasmodium cathemerium SEIAUR01
Plasmodium gallinaceum GALLUS01
Plasmodium sp. pGRW9
Plasmodium circumflexum TURDUS1
Plasmodium circumflexum SW5
Plasmodium unalis TFUS06
Plasmodium vaughani SYA1T05
Plasmodium parahexamerium ALEDIA02
Plasmodium sp. LK6
Plasmodium multivacuolaris ANLA107
Plasmodium juxtanucleare GALLUS02
Plasmodium tyanggang PYCJCC01
Plasmodium sp. PACCTAC01
Plasmodium paranucleophilum MYCAME02
Plasmodium nucleophilum DENPET03
Plasmodium ashfordi GRW02
Plasmodium delichoni COLL6
Plasmodium homocircumflexum COLL4
Plasmodium sp. SYAT24
Plasmodium elongatum GRW06
Plasmodium sp. ATTRU5
Plasmodium matalhum LINN1
Plasmodium lutzi TFUS05
Plasmodium lutzi DIGLAF01
Plasmodium lutzi DIGCYA08
Plasmodium lutzi DIGLAF02

B)

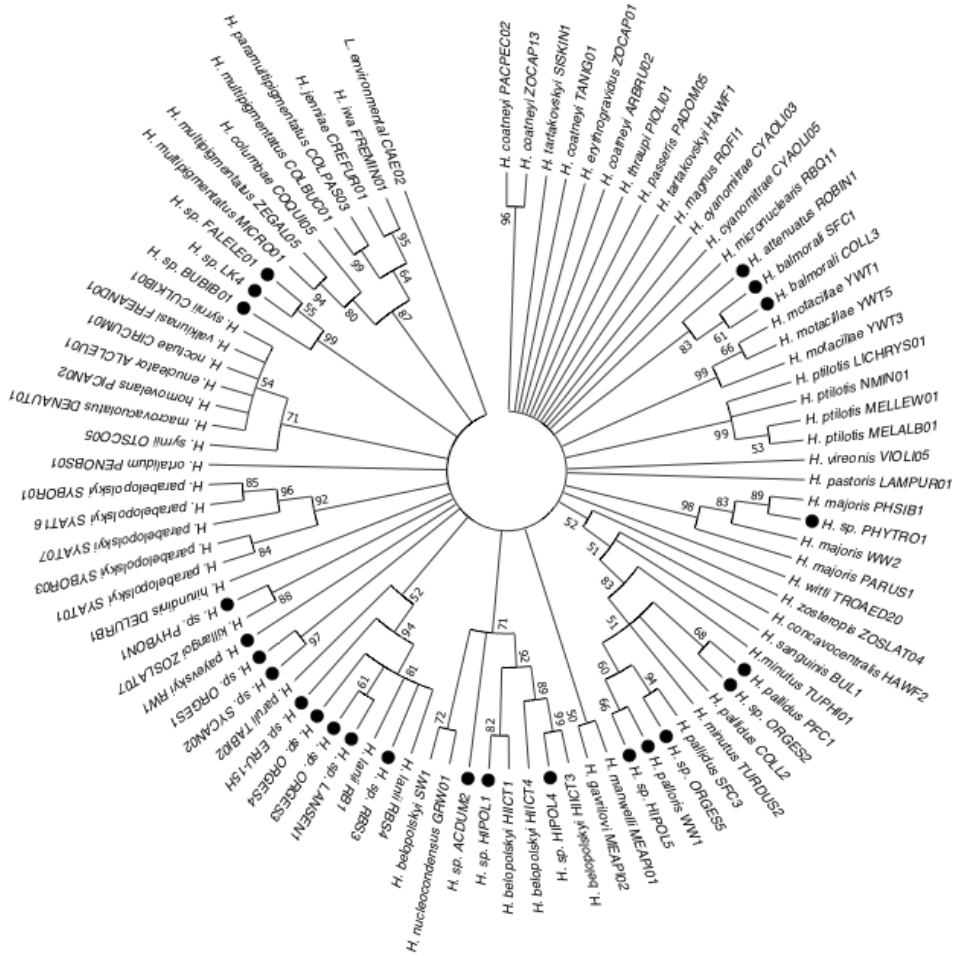


Fig. 1. Bootstrap consensus tree inferred from 100 replicated for *Plasmodium* (A) and *Haemoproteus* (B) lineages found in this study (black dots) with respect to available sequences from known morphospecies deposited in MalAvi (Bensch *et al.* 2009).

The success of transmission of a particular parasite lineage to a new host depends to a large extent on the susceptibility of the host, but also on the compatibility between blood-feeding insects and parasite lineages (Beerntsen *et al.* 2000; Martínez-de la Puente *et al*. 2011). Indeed, only a fraction of the parasites that contact with potential vectors are effectively transmitted (Gutiérrez-López *et al.* 2016), since this process may be hampered

by environmental, behavioral, genetic, and physiological factors limiting the development of parasites in blood-sucking insects (Beerntesen *et al.* 2000; Molina-Cruz *et al.* 2013a). For example, in human malaria, it has been found that the immune system of its natural vector, the mosquito Anopheles gambiae, is able to eliminate some strains of *Plasmodium* falciparum, while other strains can evade this immune barrier through the function of a particular parasite gene (Molina-Cruz *et al.* 2012, 2013a). However, some blood parasites may overcome genetic and physiological barriers of new, often evolutionarily distant vectors, as has been shown for different avian *Plasmodium* spp. transmitted by anopheline and culicine mosquitoes under laboratory conditions (Molina-Cruz, Lehmann, & Knöckel, 2013b and references therein), but also for *Plasmodium* vivax and different Anopheles species in natural environments (Joy *et al.* 2008). The process of adaptation to a new vector following environmental changes may be fuelled by the high evolutionary potential of blood parasites (Bensch et al. 2004; Joy *et al.* 2008) and fast mutations rates at some loci, such as those involved in the evasion of vector immune system (Molina-Cruz *et al.* 2015). In the parasite-vector arms race and from the parasite point of view, the benefits of adapting to a new –likely more abundant– vector must exceed the costs ensuing, as may be the reduced transmission efficiency in the original vector (Cohuet et al. 2010). On the other hand, the infection-induced fitness costs should not reduce vector survival as to prevent parasite transmission (Frank & Schmid-Hempel, 2008). Although studies dealing with this issue in the louse fly-*Haemoproteus* system are very few, Waite *et al.* (2012) showed that survival and fecundity of female *Pseudolynchia canariensis* flies decreased when feeding on birds infected by *H. columbae*, although these flies effectively transmit the parasite. We do not know to what extent the infections by blood parasites may impose fitness costs to *O. gestroi* and whether the ecological and evolutionary processes associated may affect susceptibility to the parasite and potential levels of transmission (Tripet *et al*. 2009). In any case, in a novel habitat, the continuous interaction between blood parasites and highly abundant potential vectors, within which extensive reproduction may occur at early stages of coadaptation (Ewald, 1983) would increase the likelihood of vector switching.

The contact rates between infected and new hosts are largely influenced by the feeding preference of insects and their host specificity (Gager *et al.* 2008; Malmqvist *et al*. 2004; Medeiros *et al*. 2013; Whiteman *et al*. 2006). Louse flies of the genus Ornitophila

include only two species, *O. metallica* and *O. gestroi*. While *O. metallica* has been recorded in different bird species and geographic regions (Maa, 1969), *O. gestroi* had been exclusively reported in three Falco species: Eleonora's Falcons, Common and Lesser kestrels (Beaucournu *et al.* 1985; Gangoso *et al.* 2010; Walter, 1979). Despite the clear specificity of these obligate ectoparasites, we found molecular evidence that *O. gestroi* also fed on passerines hunted by Eleonora's falcons. This fact may increase the contact rate with blood parasites carried by these bird species and hence, the likelihood of parasite spillover. But the question arises as to how the louse flies become infected. Migratory birds are hunted over the ocean by male Eleonora's falcons and subsequently transported to the breeding colony from varying distances of up to 50 km (Viana *et al.* 2016). During this travel, preys are attached to the falcon's body (Fig. 2) and louse flies have then the opportunity to feed on the immobilized –usually still alive– bird prey. The high prevalence and diversity of lineages found in louse flies suggest that this opportunistic feeding behavior is rather common. After that, louse flies may be able to reach nestlings from adult birds or even stored preys. In support of this possibility, Levin & Parker (2014) reported the ability of both infected and uninfected louse flies to move between nestlings within a bird colony. In addition, louse flies may be involved in the transmission of *Haemoproteus* parasites between distantly related species as in the case of seabirds (i.e. frigates) and passerines (Jaramillo *et al.* 2017; Santiago-Alarcon *et al.* 2014). The alternative hypothesis that louse flies are carried by the migratory passerines is unlikely, as this louse fly species, to our knowledge, have not been recorded in any other bird different than the above mentioned Falco species.

Fig. 2. Male Eleonora's falcon carrying a hunted passerine bird, which is attached to the falcon's body during transport.

In spite of differences in the sample sizes analyzed, we found a moderate similarity between the parasite lineages isolated from louse flies and bird preys. The commonest parasites of louse flies, i.e. the *Haemoproteus* lineages HIPOL1 and PFC1 were found in the more frequent species hunted by Eleonora's falcons, i.e. the European pied flycatcher and the Common whitethroat (see Tables 1 and 2). These findings together with the fact that most parasite lineages isolated from louse flies correspond to lineages infecting Passeriformes (see Table S1) support the hypothesis that the origin of the parasites isolated from *O. gestroi* are preys hunted by falcons. The phylogenetic analyses also showed that the parasite lineages found in louse flies were intermingled with lineages typical from Passeriformes. Moreover, the new lineages isolated from louse flies in this study were closely related to lineages of *H. pallidus* and *H. lanii*, both parasites of passeriforms (according to MalAvi, Bensch *et al.* 2009), including species recorded as prey of Eleonora's falcons (see Table 2 and Table S1). This suggests that louse flies are in continuous contact

with a diverse array of parasite lineages through the occasional feeding on Eleonora's falcons' preys. The *Haemoproteus* parasites infecting passerine birds across the globe belong to the subgenus Para*Haemoproteus*, which are transmitted by Culicoides (Beadell *et al.* 2006; Martinsen *et al.* 2008). In contrast, louse flies are thought to transmit *Haemoproteus* of the subgenus *Haemoproteus*, which infect Columbiformes and some seabird species (i.e. Suliformes, Charadriiformes) and a passerine (*Myiarchus magnirostris*) from the Galápagos archipelago (Levin *et al.* 2011; Sari *et al.* 2013; Valkiūnas *et al.* 2010). This suggests that the ecological factors associated to insularity may challenge the evolutionary relationships between geographically restricted hosts, potential insect vectors and avian Haemosporidians. In our case, it is possible that infections of louse flies do not contribute to parasite transmission beyond limiting parasite spread through the infection of a non-competent vector. The finding of parasites in the head-thorax of louse flies suggests that parasites are able to cross some barriers and survive to some extent within the louse flies. However, since we did not look for sporozoites, we cannot rule out that we amplified abortive infections in these hippoboscids (Valkiūnas, 2011; Valkiūnas *et al.* 2014), which would thus be dead-end invertebrate hosts of these parasites or near-successful events of vector switching.

It has been proposed that the main filter hampering the spread of haemosporidian parasites across bird species is in the parasite-host compatibility, since the vector offers a wide diversity of parasites, but only a few succeed (Medeiros *et al.* 2013). A newly colonized host represents a novel habitat for the parasite, probably having a new blood cellular and immunological profile that can hamper the ability of the parasite to invade host cells or lead to abortive development of the parasite in the tissue stage (Olias *et al.* 2011; Valkiūnas *et al.* 2014). For instance, Jaramillo *et al.* (2017) found that the introduced *H. multipigmentatus,* a parasite thought to be specific to columbiform birds (Valkiūnas *et al.* 2010) was able to infect six different species of passerines co-occurring with the parasite main host, i.e. the endemic Galapagos dove. However, and in spite of the successful spillover from introduced rock pigeons (*Columba livia*) to doves and the subsequent spillover from doves to passerines, the absence of parasite gametocytes in passerine bird blood suggest that these are not competent hosts for this *Haemoproteus* lineage (Jaramillo *et al.* 2017). Likewise, Moens *et al.* (2016) found gametocytes of the generalist *H. witti*

only in Andean hummingbirds, but not in passerines being likely infected by the parasite spillover. Molecular detection of stages of parasites including sporozoites in the bird peripheral blood could explain these results (Valkiūnas *et al*. 2009).

Beyond the filtering effects exerted by parasite-vector compatibility and parasite-host compatibility, the host immune system could also prevent falcons to become infected by new parasite lineages. Alternatively, we cannot exclude a scenario of rare transmission events associated with high parasite virulence, with infected hosts rapidly purged by high mortality (Poulin, 2006). The lack of parasites in Eleonora's falcon nestlings (Gutiérrez-López *et al.* 2015a) and the mismatch between the lineages isolated here and those previously found in adult falcons (Gangoso *et al.* 2016) support the absence of successful transmission to this raptor species. This fact is observed despite louse flies enter in contact with hemosporidian parasites coming from a wide taxonomic range of avian species. This could be due to falcons' resistance to infection, the inability of parasites to develop in these phylogenetically distant species, and/or the inability of some hemosporidian lineages to complete their development in the louse flies.

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Electronic Supplementary Material

Table S1. *Haemoproteus* and *Plasmodium* parasite lineages found in this study. The different avian host where these lineages were isolated according to MalAvi public database (Bensch et al. 2009) are shown. The different continents are abbreviated as: "Af" = Africa, "Am" = America, "As" = Asia, "Eu" = Europe, and "Oce" = Oceania. The host species recorded as prey of Eleonora's falcons (*Falco eleonorae*) on the Canary Islands are highlighted with an *.

Parasites	Bird hosts					*F. eleonorae*
Parasite lineage	**Order**	**Family**	**Genus**	**Species**	**Continent**	**Prey**
Haemoproteus sp. ACDUM2	Passeriformes	Sylviidae	Acrocephalus	*A. agricola*	As, Eu	
				A. dumetorum	As	
			Hippolais	*H. polyglotta*	Eu	*
			Sylvia	*S. communis*	Eu	*
Haemoproteus sp. HIPOL4	Passeriformes	Sylviidae	Hippolais	*H. polyglotta*	Eu	*
Haemoproteus sp. LANSEN1	Passeriformes	Laniidae	Lanius	*L. senator*	Eu	*
		Muscicapidae	Ficedula	*F. hypoleuca*	Eu	*
Haemoproteus sp. HIPOL5	Passeriformes	Phylloscopidae	Phylloscopus	*P. bonelli*	Eu	*
		Sylviidae	Hippolais	*H. polyglotta*	Eu	*
Haemoproteus attenuatus ROBIN1	Passeriformes	Laniidae	Lanius	*L. senator*	Eu	*
		Turdidae	Erithacus	*E. rubecula*	Af, As, Eu	*
			Luscinia	*L. luscinia*	As, Eu	
				L. megarhynchos	Eu	*
			Saxicola	*S. rubetra*	Af, Eu	*
Haemoproteus balmorali COLL3	Passeriformes	Muscicapidae	Ficedula	*F. albicollis*	Eu	
				F. hypoleuca	As	
				F. speculigera	Af	
			Muscicapa	*M. striata*	As	
Haemoproteus balmorali SFC1	Passeriformes	Muscicapidae	Muscicapa	*M. striata*	Af, As, Eu	*
		Paridae	Cyanistes	*C. caeruleus*	Eu	
Haemoproteus sp. ERU-15H	Passeriformes	Laniidae	Lanius	*L. collurio*	Eu-As	
Haemoproteus sp. HIPOL1	Passeriformes	Muscicapidae	Ficedula	*F. hypoleuca*	Eu	*
		Sylviidae	Hippolais	*H. icterina*	Eu	
				H. polyglotta	Eu, Af	*
			Sylvia	*S. communis*	Eu	*
		Ploceidae	Ploceus	*P. nigricollis*	Af	
Haemoproteus pallidus PFC1	Passeriformes	Fringillidae	Coccothraustes	*C. coccothraustes*	As	
		Muscicapidae	Ficedula	*F. albicollis*	Eu	
				F. hypoleuca	Af, As, Eu	*
		Phylloscopidae	Phylloscopus	*P. trochilus*	Eu	*
		Sylviidae	Acrocephalus	*A. paludicola*	Eu	*
			Sylvia	*S. communis*	Eu	*
Haemoproteus palloris WW1	Anseriformes	Anatidae	Cygnus	*C. olor*	Eu	
	Passeriformes	Muscicapidae	Ficedula	*F. hypoleuca*	As	
		Estrildidae	Uraeginthus	*U. bengalus*	Af	
		Panuridae	Panurus	*P. biarmicus*	As	
		Paridae	Cyanistes	*C. caeruleus*	Eu	
			Poecile	*P. montanus*	As	
		Ploceidae	Euplectes	*E. macroura*	Af	
		Phylloscopidae	Phylloscopus	*P. trochilus*	Af, As, Eu	*
		Sylviidae	Acrocephalus	*A. scirpaceus*	As	
			Sylvia	*S. borin*	Eu	*
	Upupiformes	Upupidae	Upupa	*U. epops*	As	
Haemoproteus payevsyi RW1	Passeriformes	Laniidae	Lanius	*L. meridionalis*	Eu	
		Cinclidae	Cinclus	*C. cinclus*	Eu	
		Cisticolidae	Cisticola	*C. nigriloris*	Af	
		Sylviidae	Acrocephalus	*A. baeticatus*	Af	
				A. scirpaceus	As, Eu	*
				A. schoenobaenus	Eu	
				A. palustris	As	
		Tudidae	Luscinia	*L. svecica*	Eu	*
Haemoproteus sp. RBS3	Passeriformes	Laniidae	Lanius	*L. collurio*	Eu	
				L. minor		
				L. senator	Eu	*
Haemoproteus sp. ORGES1						

Haemoproteus sp. ORGES2						
Haemoproteus sp. ORGES3						
Haemoproteus sp. ORGES4						
Haemoproteus sp. ORGES5						
Haemoproteus sp. PHYBON1	Passeriformes	Phylloscopidae	Phylloscopus	*P. bonelli*	Eu	*
Haemoproteus sp. PHYTRO1	Passeriformes	Phylloscopidae	Phylloscopus	*P. trochilus*	Eu	*
Haemoproteus sp. SYCAN02	Passeriformes	Sylviidae	Sylvia	*S. cantillans*	Eu	*
Plasmodium Aftru5	Passeriformes	Turdidae	Luscinia	*L. svecica*	As	
			Turdus	*T. merula*	Eu, Af	
				T. pelios	Af	
		Muscicapidae	Erithacus	*E. rubecula*	Eu	*
	Psitaciformes	Psittacidae	Melopsittacus	*M. undulatus*	Oce	
Plasmodium sp. LK6	Falconiformes	Falconidae	Falco	*F. eleonorae*	Eu	
				F. naumanni	Eu	
	Passeriformes	Alaudidae	Galerida	*G. cristata*	Af	
		Fringillidae	Emberiza	*E. cirlus*	Af	
			Serinus	*S. canarius*	Eu	
		Motacillidae	Anthus	*A. berthelotti*	Eu	
				A. campestris	Eu	*
		Muscicapidae	Phoenicurus	*P. moussieri*	Af	
		Paridae	Periparus	*P. ater*	Af	
			Cyanistes	*C. teneriffae*	Af	
		Phylloscopidae	Phylloscopus	*P. canariensis*	Eu	
		Sylviidae	Sylvia	*S. conspicillata*	Af, Eu	
				S. melanocephala	Af, Eu	
Plamodium sp. MOALB1	Passeriformes	Alaudidae	Alauda	*A. arvensis*	Eu	
		Motacillidae	Motacilla	*M. alba*		*
				M. flava	Eu	*
Plasmodium relictum GRW11	Charadriiformes	Scolopacidae	Philomachus	*P. pugnax*	Eu	
	Gruiformes	PhAsnidae	Lophophorus	*L. impejanus*	As	
			Chrysolophus	*C. amherstiae*	As	
	Passeriformes	Alaudidae	Alauda	*A. arvensis*	Eu	
		Certhiidae	Troglodytes	*T. troglodytes*	Eu	
		Corvidae	Corvus	*C. corone*	As, Eu	
				C. macrorhynchos	As	
			Garrulus	*G. glandarius*	As	
		Fringiliidae	Carduelis	*C. carduelis*	As	
			Emberiza	*E. cirlus*	Eu	
			Fringilla	*F. coelebs*	As, Af, Eu	
			Pyrrhula	*P. pyrrhula*	As	
		Muscicapidae	Ficedula	*F. albicollis*	Eu	
			Phoenicurus	*P. ochruros*	Eu	*
		Hirundinidae	Delichon	*D. urbicum*	Af, Eu	
				D. dasypus	As	
		Laniidae	Lanius	*L. collurio*	Eu	
		Passeridae	Passer	*P. domesticus*	Af, Eu	
				P. hispaniolensis	Af, Eu	
				P. montanus	As, Eu	
		Pariidae	Cyanistes	*C. caeruleus*	Eu	
				C. teneriffae	Af	
			Parus	*P. major*	As, Eu	
			Periparus	*P. ater*	Af	
		Pycnonotidae	Pycnonotus	*P. capensis*	Af	
			Acrocephalus	*A. agricola*	Eu	
				A.arundinaceus	Eu	
				A. scirpaceus	Eu	*
			Cettia	*C. cetti*	Eu	

		Sylviidae	Sylvia	*S. atricapilla*	Eu	
				S. borin	As, Af, Eu	*
				S. cantillans	Eu	*
				S. communis	As	*
				S. conspicillata	Eu	
				S. curruca	As	
				S. melanocephala	Eu	
				S. nisoria	As	
		Turdidae	Erithacus	*E. rubecula*	Eu	*
			Luscinia	*L. luscinia*	Eu	
				L. svecica	Eu	*
			Saxicola	*S. rubetra*	As	
	Anseriformes	Anatidae	Anas	*A. acuta*	Eu	
			Marmaronetta	*M. angustirostris*	Eu	
	Charadriiformes	Laridae	Larus	*L. argentatus*	Eu	
				L. cachinnans	Eu	
				L. mongolicus	As	
		Recurvirostridae	Himantopus	*H. himantopus*	Eu	
		Scolopacidae	Gallinago	*G. gallinago*	Eu	
	Ciconiiformes	Ardeidae	Botaurus	*B. stellaris*	As	
			Bubulcus	*B. ibis*	Eu	
		Ciconiidae	Ciconia	*C. ciconia*	Eu	
	Columbiformes	Columbidae	Columba	*C. livia*	Eu	
	Galliformes	PhAsnidae	Gallus	*G. gallus*	Eu	
			Lophophorus	*L. impejanus*	As	
			Perdix	*P. perdix*	Eu	
			Tragopan	*T. temminckii*	As	
	Gruiformes	Gruidae	Grus	*G. nigricollis*	As	
		Alaudidae	Alauda	*A. arvensis*	Eu	
		Certhiidae	Certhia	*C. brachydactyla*	Af, Eu	
			Troglodytes	*T. aedon*	S Am	
				T. troglodytes	Af, As, Eu	
		Corvidae	Corvus	*C. corone*	As, Eu	
				C. macrorhynchos	As	
			Cyanopica	*C. cooki*	Eu	
			Garrulus	*G. glandarius*	As, Eu	
			Pica	*P. pica*	Eu	
		Estrildidae	Estrilda	*E. astrild*	Eu	
		Fringillidae	Carduelis	*C. chloris*	Af, As, Eu	
				C. carduelis	As, Eu	
				C. spinus	As	
			Carpodacus	*C. erythrinus*	As, Eu	
			Coccothraustes	*C. coccothraustes*	As	
			Conirostrum	*C. cinereum*	S Am	
			Emberiza	*E. calandra*	As	
				E. cia	Eu	
				E. cirlus	Eu	
				E. citrinella	Oce	
				E. elegans	As	
				E. godlewskii	As	
				E. hortulana	As	
				E. tahapisi	Af	
			Fringilla	*F. coelebs*	Af, As, Eu	
			Loxia	*L. curvirostra*	As	
			Pyrrhula	*P. pyrrhula*	Eu	
			Serinus	*S. canaria*	Eu	
				S. serinus	Af, Eu	
			Zonotrichia	*Z. capensis*	S Am	
		Furnariidae	Phleocryptes	*P. melanops*	S Am	
		Hirundinidae	Delichon	*D. urbicum*	Af, Eu	
		Laniidae	Lanius	*L. collaris*	Af	
				L. senator	Eu	*

Plasmodium relictum SGS1 (Rinshi-1)	Passeriformes	Motacillidae	Motacilla	*M. flava*	Eu	*
		Muscicapidae	Cercotrichas	*C. coryphoeus*	Af	
				C. galactotes	Af	*
				C. podobe	Af	
			Ficedula	*F. albicollis*	Eu	
				F. hypoleuca	As, Eu	*
			Muscicapa	*M. striata*	Af, As	*
			Oenanthe	*O. oenanthe*	Af	*
			Phoenicurus	*P. moussieri*	Af	
				P. ochruros	Eu	*
				P. phoenicurus	As, Eu	*
		Paridae	Cyanistes	*C. caeruleus*	As, Eu	
				C. teneriffae	Af	
			Lophophanes	*L. cristatus*	Eu	
			Parus	*P. major*	Af, As, Eu	
				P. venustulus	As	
				P. palustris	As	
			Periparus	*P. ater*	Af, Eu	
			Poecile	*P. montanus*	As	
				P. varius	As	
		Passeridae	Passer	*P. domesticus*	Af, As, Eu, Oce	
				P. griseus	Af	
				P. hispaniolensis	Eu	
				P. luteus	Af	
				P. melanurus	Af	
				P. montanus	As, Eu	
				P. rufocinctus	Af	
			Prunella	*P. modularis*	As	
		Phylloscopidae	Phylloscopues	*P. bonelli*	Eu	*
		Ploceidae	Euplectes	*E. orix*	Af	
			Ploceus	*P. capensis*	Af	
				P. melanocephalus	Eu	
				P. velatus	Af	
		Pycnonotidae	Microscelis	*M. amaurotis*	As	
			Pycnonotus	*P. capensis*	Af	
			Hypsipetes	*H. amaurotis*	As	
		Sittidae	Sitta	*S. europaea*	As	
		Sturnidae	Sturnus	*S. cineraceus*	As	
				S. tristis	As, Oce	
		Sylviidae	Acrocephalus	*A. agricola*	Eu	
				A. arundinaceus	Eu	
				A. palustris	As	
				A. schoenobaenus	Af	
				A. scirpaceus	Af, As, Eu	*
			Cettia	*C. cetti*	As, Eu	
			Hippolais	*H. polyglotta*	Eu	*
			Sylvia	*S. atricapilla*	Eu	
				S. borin	Af, As, Eu	*
				S. communis	As, Eu	*
				S. curruca	Af, As	
				S. deserticola	Af	
				S. melanocephala	Af, Eu	
				S. nisoria	Eu	
				S. undata	Eu	
		Turdidae	Erithacus	*E. rubecula*	As, Eu	*
			Luscinia	*L. svecica*	Eu, As	*
			Monticola	*M. saxatilis*	As	
			Saxicola	*S. rubetra*	Af, Eu	*
				S. maura	As	
			Turdus	*T. merula*	Eu	
				T. viscivorus	Af	
		Tyrannidae	Savornis	*S. nigricans*	S Am	
			Serpophaga	*S. cinerea*	S Am	

	Procellariformes	Procellariidae	Pachyptila	*P. belcheri*	S Am	
	Spheniciformes	Spheniscidae	Spheniscus	S. humboldti	As	
	Strigiformes	Strigidae	Athene	*A. noctua*	Eu	
	Trochiliformes	Trochilidae	Amazilia	*A. chionogaster*	S Am	
			Colibri	*C. coruscans*	S Am	
Plasmodium sp. COLL1	Passeriformes	Fringillidae	Emberiza	*E. cia*	Eu	
				E. cirlus	Eu	
		Motacillidae	Anthus	*A. campestris*	Eu	*
			Motacilla	*M. flava*	Eu	*
		Paridae	Cyanistes	*C. caeruleus*	Eu	
		Passeridae	Passer	*P. domesticus*	Eu	
				P. hispaniolensis	Eu	
		Ploceidae	Euplectes	*E. orix*	Af	
		Sylviidae	Acrocephalus	*A. paludicola*	Eu	*
			Locustella	*L. luscinioides*	Eu	
			Sylvia	*S. atricapilla*	Eu	
Plasmodium sp. GRW9	Passeriformes	Cisticolidae	Camaroptera	*C. brachyura*	Af	
		Dicruridae	Dicrurus	*D. adsimilis*	Af	
			Rhipidura	*R. rufifrons*	Oce	
		Estrildidae	Estrilda	*E. astrild*	Af	
				E. melpoda	Af	
			Lonchura	*L. cucullata*	Af	
			Pyrenestes	*P. ostrinus*	Af	
			Spermophaga	*S. haematina*	Af	
			Uraeginthus	*U. angolensis*	Af	
		Fringillidae	Crithagra	*C. capistrata*	Af	
			Serinus	*S. mozambicus*	Af	
		Hirundinidae	Delichon	*D. urbicum*	Eu	
			Hirundo	*H. rustica*	Eu	
			Phedina	*P. borbonica*	Af	
			Psalidoprocne	*P. albiceps*	Af	
		Laniidae	Lanius	*L. collurio*	Eu	
		Motacillidae	Motacilla	*M. flaviventris*	Af	
		Muscicapidae	Copsychus	*C. albospecularis*	Afica	
			Cossypha	*C. heuglini*	Af	
				C. niveicapilla	Af	
			Ficedula	*F. albicollis*	Eu	
				F. hypoleuca	As, Eu	*
				F. semitorquata	Eu	
			Muscicapa	*M. olivascens*	Af	
			Pogonocichla	*P. stellata*	Af	
		Nectariniidae	Chalcomitra	*C. rubescens*	Af	
			Cinnyris	*C. chloropygius*	Af	
				C. fuelleborni	Af	
				C. mediocris	Af	
			Cyanomitra	*C. olivacea*	Af	
				C. verticalis	Af	
			Deleomis	*D. fraseri*	Af	
			Hedydipna	*H. collaris*	Af	
			Nectarinia	*N. chloropugia*	Af	
		Passeridae	Gymnoris	*G. supercilliaris*	Af	
		Philepittidae	Philepitta	*P. castanea*	Af	
		Platysteiridae	Batis	*B. mixta*	Af	
		Ploceidae	Euplectes	*E. hordeaceus*	Af	
			Foudia	*F. hypoleuca*	Af	
				F. madagascariensis	Af	
			Malimbus	*M. nitens*	Af	
			Ploceus	*P. cucullatus*	Af	
				P. nelicourvi	Af	
			Quelea	*Q. quelea*	Af	
				A. chlorigula	Af	

		Pycnonotidae	Andropadus	*A. gracilis*	Af	
				A. latirostris	Af	
				A. masukuensis	Af	
				A. milanjensis	Af	
				A. virens	Af	
			Bernieria	*B. madagascariensis*	Af	
			Bleda	*B. eximius*	Af	
				B. notatus	Af	
				B. syndactylus	Af	
			Criniger	*C. calurus*	Af	
				C. chloronotus	Af	
			Hypsipetes	*H. madagascariensis*	Af	
			Nicator	*N. chloris*	Af	
			Phyllastrephus	*P. icterinus*	Af	
			Pycnonotus	*P. barbatus*	Af	
		Sylviidae	Acrocephalus	*A. arundinaceus*	Eu	
			Bradypterus	*B. baboecala*	Af	
			Nesillas	*N. typica*	Af	
			Sylvia	*S. borin*	Eu	*
		Timallidae	Modulatrix	*M. stictigula*	Af	
		Turdidae	Alethe	*A. fuelleborni*	Af	
				A. poliocephala	Af	
			Neocossyphus	*N. fraseri*	Af	
				N. poensis	Af	
				N. rufus	Af	
			Saxicola	*S. rubetra*	Eu	*
				S. torquata	Af	
			Sheppardia	*S. sharpei*	Af	
			Stiphrornis	*S. erythrothorax*	Af	
			Zoothera	*Z. camaronensis*	Af	
Plasmodium sp. SYAT24	Passeriformes	Sylviidae	Sylvia	*S. atricapilla*	Eu	
Plasmodium vaughani SYAT05 (Rinshi-11)	Columbiformes	Columbidae	Hemiphaga	*H. novaeseelandiae*	Oce	
	Passeriformes	Alaudidae	Alauda	*A. arvensis*	Eu	
		Cinclidae	Cinclus	*C. cinclus*	Eu	
		Fringillidae	Fringilla	*F. coelebs*	Eu	
		Melipgagidae	Anthornis	*A. melanura*	Oce	
		Muscicapidae	Ficedula	*F. parva*	Eu	
		Paridae	Cyanistes	*C. caeruleus*	Eu	
			Parus	*P. major*	Eu	
		Petroicidae	Petroica	*P. australis*	Oce	
				P. macrocephala	Oce	
		Sturnidae	Sturnus	*S. unicolor*	Eu	
		Sylviidae	Cettia	*C. cetti*	Eu	
			Sylvia	*S. atricapilla*	Eu	
				S. borin	Eu	*
				S. communis	Eu	*
				S. malanocephala	Eu	
		Turdidae	Erythacus	*E. rubecula*	Eu	*
			Saxicola	*S. maura*	As	
			Turdus	*T. merula*	Af, As, Eu, Oce	
				T. migratorius	N Am	
				T. pelios	Af	
				T. philomelos	Eu	
				T. viscivorus	Af	
		Zosteropidae	Zosterops	*Z. lateralis*	Oce	
	Psittaciformes	Psittacidae	Melopsittacus	*M. undulatus*	Oce	

Discussion

The transmission of vector-borne pathogens embraces several complex processes where biotic and abiotic factors modulate the evolutionary arms race between hosts, vectors and pathogens (Thompson 1998, de Roode *et al*. 2008). Most studies on the transmission of vector-borne pathogens of health concern have been performed under laboratory conditions. Although these studies have provided valuable information, they may, however, under-represent the diversity of interactions found under natural conditions (Anderson & May 1992, Grenfell & Dobson 1995, Beerntsen *et al*. 2000). Therefore, to better understand the actual transmission networks of pathogens, further studies should consider the diversity of parasite species/lineages and vector/host species as well as the different factors potentially influencing their interactions. During the course of this thesis, by combining knowledge and methodologies from different disciplines including ecology, virology and epidemiology (e.g. sampling of wild birds and mosquitoes, mosquito rearing and maintenance in climatic chambers, taxonomic identification, traditional analyses of blood smears, molecular analyses for the detection and identification of parasite lineages, experiments under semi-natural and safety laboratory conditions and different statistical approaches), I assessed the relative importance of factors such as host, mosquito, and pathogen strain/species and levels of parasite load, on the transmission of two important vector-borne pathogens, i.e. the avian malaria parasites *Plasmodium* and the Zika virus.

Despite its crucial importance for human and wildlife health, basic information necessary to quantify the transmission risk of most mosquito-borne pathogens is currently lacking. The interactions between mosquitoes and vertebrate hosts modulate the pathogens' transmission success (Ross, 1911; Macdonald, 1955). In this regard, mosquito feeding behaviour represents the first step for pathogen transmission, which may be influenced by both mosquito (i.e. mosquito species, life cycle, **chapter 1**) and host-related traits (i.e. odour, heat, morphological traits, behaviour, and intensity of infection by vector-borne pathogens; Takken & Verhuklst, 2013; Cornet *et al*. 2013; Yan *et al*. 2017, 2018). However, most studies have focused on the study of the inter-specific variation in host´s traits affecting their interactions with vectors (Kilpatrick *et al*. 2006; Rizzoli *et al.* 2015), while host intra-specific variability has been traditionally neglected. Therefore, identifying the potential factors causing differences in the biting patterns of different mosquito-host

assemblages, including those occurring at the intra-specific level, would allow us to identify the super-spreaders, which is essential to understand the transmission risk of mosquito-borne pathogens in the wild. In this thesis, using two mosquito species with different feeding patterns and two bird hosts (**chapter 1**), I found clear differences in the biting rate of the two mosquitoes, which could be in turn influenced by hosts' traits such as body mass and gender, although these effects may differ depending on the specific mosquito-host assemblage. Therefore, the biting patterns of mosquitoes seem to be far from being generalizable. Recent studies have found support for the potential role of some factors, such as the avian infection status by mosquito-borne pathogens and the bird metabolic rate in the variation in mosquito biting behavior (Yan *et al*. 2017, 2018). The mechanisms underlying this differential susceptibility of hosts to mosquito bites requires further attention, but could be mediated by differences in the emision of cues (e.g. olfactory cues) between individuals (Takken & Verhuklst 2013; Robinson *et al*. 2018). In addition, the discrepancies found between studies regarding the relative importance of different host traits on mosquito's feeding preferences could be due to methodological differences. While some studies identified the origin of the blood meals of mosquitoes trapped under natural conditions (Burket-Cadena *et al.* 2014), others identifed the mosquito feeding preferences by comparing the attraction of mosquitoes to different cues (Lalubin *et al.* 2012). Exposing host individually or in pairs, may also partilly affect the conclusions obtained in different studies. For example, in contrast to what found in studies that identified the biting preferences of mosquitoes by exposing them to both experimentally infected and un-infected birds (Cornet *et al*. 2013), I did not find any relationship between host infection status by avian malaria and the feeding preference of mosquitoes after exposing them to naturally infected single host (**chapter 1**). Therefore, methodological differences may explain the different results of these studies.

In epidemiological studies, after identifying the potential factors affecting the mosquito-host interactions (i.e. biting rates), the next essential step is to understand the factors that determine the development of pathogens in mosquitoes (Ross, 1911, Macdonald, 1955). Once ingested, the mosquito midgut acts as the first barrier for parasite development, representing an important selective force for pathogens (Abraham *et al*. 2004). In addition, pathogens must evade the insect's immune system to complete its

development to finally reach the mosquito salivary glands for its successful transmission. Under natural conditions, mosquitoes can feed on birds infected by a diversity of pathogens, including some that can infect and develop in the insect species, but also other pathogens that could not develop in this particular insect species and require a different vector species. However, information on the spectrum of the competent vectors for the transmission of pathogens affecting wildlife is certainly scarce, as may be the case of insect species involved in the transmission of avian malaria parasites and related Haemosporidians. The identification of intermediate non-infective stages of the parasite (i.e. oocysts) or parasite DNA through molecular techniques have been traditionally used to assess the potential mosquito vectors (Njabo *et al*. 2011, Ferraguti *et al*. 2013, Pigeault *et al*. 2015). However, although these methods provide valuable information, they are not suitable for the correct identification of the actual competent vectors of these pathogens (Gamage-Mendis *et al*. 1993; Valkiūnas *et al*. 2018), because often DNA correspond to forms of the parasite that can not be effectively transmitted. In this thesis, I used a more robust method based on the analysis of the mosquito's saliva to assess the vector competence of different mosquito species for different parasite species/lineages. The results obtained here support that from two phylogenetically related pathogens (i.e. *Plasmodium* and *Haemoproteus*) only *Plasmodium* can develop in *Culex pipiens* mosquitoes feeding on infected birds (**chapter 3**). In addition, the ability of mosquito-borne pathogens to develop in mosquitoes differs between mosquito species (Palinauskas *et al*. 2016; Ciota *et al*. 2017). The capacity of a particular pathogen to develop in a certain mosquito species is the result of complex co-evolutionary processes (Leggett *et al*. 2013). Some mosquitoes are completely unable to transmit some pathogens despite being exposed to them (e.g. Zika virus and avian *Plasmodium* by *Ae. caspius*; **chapters 2 and 4**) while these same pathogens complete its life cycle in other mosquito species (e.g. Zika virus in *Ae. albopictus* and avian *Plasmodium* in *Cx. pipiens*; **chapters 2 and 4**). Differences in the vector competence of different mosquitoes for the transmission of each pathogen might be affected by a number of physiological and biochemical processes (Abraham *et al*. 2004), including the interactions of pathogens with proteins of the membrane of the mosquito midgut epithelium (Ishino *et al*. 2006, Siden-Kiamos *et al*. 2006, Povelones *et al*. 2009). The interspecific differences in the vector competence of mosquitoes for the transmission

of Zika virus **(chapter 2)**, avian *Plasmodium* (**chapter 4**) and *Haemoproteus* (**chapter 3**) could be the result of inter-specific variations in the presence/abundance of these proteins in the mosquito. Furthermore, differences in the immune responses elicited by mosquitoes against pathogens or in the insect microbiota may also affect pathogen development in the mosquito (Ramirez *et al*. 2012, Molina-Cruz *et al*. 2013), and could partially explain results found here (see **Annex 2**). In fact, previous studies have pointed to the role of *Wolbachia* bacteria in the transmission of Zika virus and avian *Plasmodium* (Hughes *et al*. 2011, Zélé *et al*. 2012, Zéle *et al*. 2014, Dutra *et al*. 2016, Aliota *et al*. 2016). Moreover, in this thesis, I also found important differences in the ability of pathogen strains/lineages to develop in the same mosquito species (i.e. Zika virus strains Puerto Rico and Cambodia by *Ae. albopictus* or avian *Plasmodium* lineages in *Cx. pipiens*) (**chapters 2 and 4**). These patterns could be due to genetic differences between strains/lineages, finally affecting the efficacy of parasite development in the mosquitoes. It is possible that both Zika virus strains and avian *Plasmodium* lineages differ in the time required to develop and reach the salivary glands, with the Zika virus strain Cambodia being faster than the Puerto Rico strain (**chapter 2**) or avian *Plasmodium* lineages of clade B (morphospecies *relictum*) being faster than those of the clade A (generically related to morphospecies *cathemerium*) (**chapter 4**)) as found by previous studies (LaPointe *et al*. 2010, Palinauskas *et al*. 2016, Ciota *et al*. 2017, Ohm *et al*. 2018). These results highlight the necessity to identify the mechanisms determining the capacity of the different pathogens to be successfully transmitted by particular mosquito species.

The parasite load of the vertebrate host may also determine the ability of the parasite to develop in the insect vector and, consequently, its transmission success. In humans, *Plasmodium* gametocytaemia (i.e. proportion of red blood cells infected by gametocytes, the sexual stage of the parasites ingested by mosquitoes) was found to be positively associated with the mosquito infection rate, supporting the association between host parasite load and transmission success (Bousema & Drakeley 2011). However, in avian *Plasmodium,* Pigeault *et al*. (2015) did not find any significant association between the host parasitaemia (i.e. proportion of red blood cells infected by the parasite, which is strongly correlated with gametocytaemia) and the prevalence of oocysts in the mosquito midgut. The results obtained in **chapter 4** support the role of host parasite load on the

infection prevalence in mosquitoes, but not this effect is only significant when analysing the final development of *Plasmodium* in mosquito saliva (**chapter 4**). However, even at very low parasite loads, undetectable by microscopy, *Plasmodium* parasites have the ability to infect and develop in the mosquitoes (Haji *et al.* 1996; Churcher *et al.* 2013; Lin *et al.* 2014). Additionally, by experimentally reducing the *Plasmodium* parasite load in the vertebrate host, I observed that this variable influenced the virulence (i.e. the cost of the pathogen infections on their host) of the pathogen on the mosquito in terms of survival probability (**chapter 5**). It is generally assumed that vector-borne parasites produce minimal costs to their vectors compared to those induced in their vertebrate hosts (Ewald & Schubert 1989). However, our results provide evidence of the impact of *Plasmodium* infections on mosquito longevity (**chapter 5**, but see **chapter 4**), providing new insights into a currently debated question (Vézilier *et al.* 2012; Pigeault & Villa 2018, **annex 2**). In addition to mosquito linked-factors, including the insect nutritional status and microbiota (**annex 2**), results from this thesis support the importance of using experimental manipulations of the avian *Plasmodium* load to identify the impact of infections in mosquitoes, as previously done in vertebrate hosts (Merino *et al*. 2000; Martínez-de la Puente *et al*. 2010; Asghar *et al*. 2015). Interestingly, in this thesis I also found support for differences according to parasite-vector assemblage on the virulence of *Plasmodium* parasites in mosquitoes (Mackinnon & Read, 2004). The use of particular lineages (especially the *Plasmodium relictum* SGS1 lineage) may partially explain the discrepancies in the effects of *Plasmodium* infection on the survival of mosquitoes found between studies (Pigeault *et al.* 2015, Pigeault & Villa 2018, Lalubin *et al.* 2014). Although differences in the level of virulence between avian *Plasmodium* lineages on bird hosts have been recorded (Lachish *et al*. 2011), its relevance for mosquito survival is currently unknown. In this thesis, I found differences in the level of virulence on mosquitoes of *Plasmodium* lineages corresponding to, at least, two morphospecies (i.e. the *Plasmodium relictum* lineages SGS1 and GRW11 and the *Plasmodium* spp. lineages PADOM01 and COLL1) (**chapter 4**). These results support the importance of considering the parasite identity in further studies of the virulence of avian *Plasmodium* parasites in mosquitoes, which is especially relevant due to the diversity of avian *Plasmodium*-mosquito species assemblages found in the wild.

The epidemiology of vector-borne pathogens depends on different factors potentially affecting pathogen transmission risk between hosts, including host density (Gubbins *et al*. 2008), host recovery rate (Macdonald, 1955) and density of vectors (Hartemink *et al*. 2009), among others (see Fig. 1 in General Introduction). Nonetheless, the transmission risk of a vector-borne pathogen is largely determined by different features of the disease system concerned, including vector daily survival rates and the probability of a vector of becoming infected after biting an infected individual (Smith *et al*. 2012). However, to the best of our knowledge, no study has addressed the effects of the last two factors on the transmission risk of avian *Plasmodium* before. In this thesis, I provided an important contribution to this regard. I found that the transmission risk of avian *Plasmodium* was strongly affected by the mortality imposed by *Plasmodium* on *Cx. pipiens* as well as by the development of *Plasmodium* parasites in the mosquito reaching the insect salivary glands (**chapters 4 and 5**). I identified the impact of these two variables on the transmission risk of *Plasmodium* parasites through the estimation of the basic reproductive ratio (R_0), an epidemiological parameter that reflects the number of secondary infections expected from an infectious individual entering into a naïve population (Ross, 1911; Macdonald, 1955). These models suggest that parasite load in the vertebrate and parasite lineage (or morphospecies) had an important impact of *Plasmodium* transmission dynamics. Nonetheless, other factors, such as the feeding preferences of mosquitoes should be also considered in epidemiological studies.

In addition to studies conducted under laboratory conditions, it is essential to identify how environmental factors may shape pathogen transmission dynamics under natural conditions (Ferraguti *et al*. 2018). I dealt with this issue in the last section of the thesis. I performed a study using the Eleonora's falcon as a study model to assess the importance of the environmental conditions as well as genetic and immunological factors in determining the dynamics of transmission of avian malaria parasites. I found that the environmental characteristics of marine habitats (i.e. strong winds, high salinity, and the scarcity of fresh water sources in which competent insect vector can develop) may determine the unsuccessful transmission of vector-borne pathogens. Using molecular screening of blood samples from birds, I found that while adult Eleonora´s falcons were infected by avian malaria parasites and related Haemosporidians (**chapters 6** and **7**) these

parasites were absent in nestlings (**chapter 6**). In addition, the identity of avian malaria parasites lineages found in the only potential insect vector present in the study area, i.e. the louse fly *Ornithophila gestroi* (**chapter 8**), support the importance of the parasite-vector assemblages for the transmission of avian malaria parasites. The positive amplification of parasite DNA from the head-thorax of louse flies (**chapter 8**) could derive from non-infective forms of the parasites, which are unable to complete their multiplicative cycle (Valkiūnas *et al*. 2005, Seblova *et al*. 2014, Martínez-de la Puente *et al*. 2011, Ferraguti *et al*. 2013). In **chapter 8**, I found that the preys hunted by the Eleonora's falcons were the source of parasites isolated in the head-thorax of louse flies. The infection of adult Elenora's falcons by avian malaria parasites of the genus *Haemoproteus* and *Plasmodium* likely took place in the wintering areas or during migration, where falcons enter in contact with competent vectors for these parasites (i.e. mosquitoes and biting midges). This model study species shows a particular trait, the presence of genetic colour polymorphism that made it suitable for assessing the importance of host genotype and associated phenotypic traits on exposure or resistance to parasites. I found that the adult falcons of dark morph had a higher prevalence of avian *Plasmodium* that pale ones, suggesting a possible unequal exposure to mosquito bites (**chapter 7**). Although in **chapter 2** I failed to find the intraspecific host-related traits that modulate the biting rate of mosquitoes when studying house sparrows and jackdaws, in **chapter 7**, the colour seems to be a host characteristic attractive to insect vectors in the Eleonora falcon, that presents a more extreme variation in coloration that the other two avian species. In fact, it has been suggested that darker colours are more attractive to mosquitoes than light colours and so, entirely dark plumages could increase host–vector contact rates (Allan *et al*. 1987). However, the different prevalence of infection found between alternative colour morphs might be also the result of immune related processes associated with the presence of specific allelic variants. In fact, a previous study showed that nestling dark Eleonora's falcons presented poorer innate and acquired immune responses than pale morphs (Gangoso *et al*. 2015).

Altogether, this thesis identified the factors that determine the successful transmission of two important vector-borne pathogens, and the impact of these factors on their transmission dynamics. With the use of a multidisciplinary approach that integrates knowledge from disciplines such as ornithology, entomology, ecology, virology, genetics,

and epidemiology, I contributed to a better understanding of the ecology of vector-borne diseases under natural conditions. This thesis also highlighted the need to better characterize the successful vector-pathogen and host-pathogen assemblages under natural conditions and the mechanisms that determine the vector competence for different pathogens. This will allow to reveal the complex transmission network of pathogens and the importance of environmental conditions in these continuously co-evolving interactions.

Conclusions

1. The magnitude and direction of the effects of the hosts' body mass, gender and the infection status by the mosquito-borne avian *Plasmodium* on the feeding patterns of mosquitoes are far from being generalizable. Only sex was associated with differences in mosquito biting rates, and this effect was only detected for *Ae. caspius*.
2. *Aedes albopictus* is a competent vector for Zika virus and its presence determine the risk of Zika virus transmission in Europe. This is especially relevant given the rapid expansion of *Ae. albopictus* in Spain, and the high number of imported Zika virus cases in the area. In contrast, the risk of Zika virus transmission by native *Ae. caspius* is considered to be extremely low.
3. *Culex pipiens* is a competent vector for avian *Plasmodium*, but it is unable to transmit *Haemoproteus* parasites. The analysis of saliva samples from potential mosquito vectors represents a useful method to determine the transmission of avian *Plasmodium* parasites in studies of vector competence.
4. There are important inter-specific and intra-specific differences in the competence of mosquitoes for the transmission of *Plasmodium* lineages. While some mosquitoes such as *Ae. caspius*, completely inhibit the parasite development, *Cx. pipiens* may play an important role in the transmission of avian *Plasmodium* parasites. Vector-parasite assemblages may modulate the transmission success of each *Plasmodium* lineage through different processes that affect the required time for parasite development and the virulence on the insect vector.
5. Avian *Plasmodium* imposes deleterious effects on mosquito survival, although the costs imposed may differ depending on the species/lineages of *Plasmodium*. Pathogen-induced costs on mosquito survival has a great impact on *Plasmodium* transmission risk.
6. Different environmental conditions shape the transmission success of vector-borne pathogens to vertebrate hosts. In insular ecosystems, the prevailing environmental conditions limit the presence of competent vectors and hence, hamper the transmission dynamics of Haemosporidian parasites.

7. Differences in host genotype and associated phenotypic traits (i.e. melanin-based colouration and immune-competence) explain the intra-specific differences in the prevalence of avian malaria parasites.

8. The lack of infection by avian malaria parasites in Eleonora's falcon nestlings and the mismatch between the lineages found in the louse flies *Ornithophila gestroi* and those found in adult Eleonora's falcons suggest that louse flies are not competent for the transmission of avian Haemosporidians infecting a wide taxonomic range of avian species.

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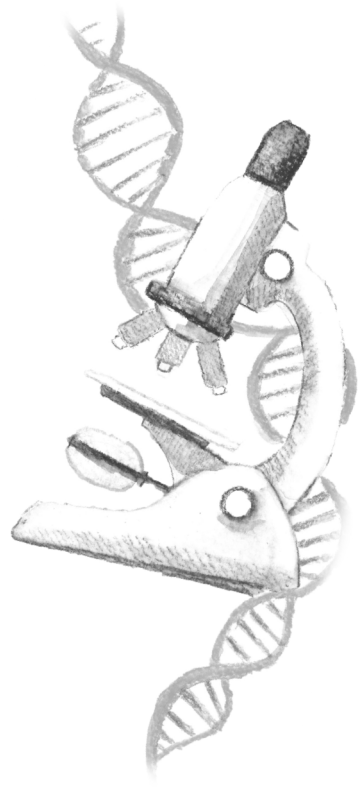
Annexes



Annex 1

Comparison of manual and semi-automatic DNA extraction protocols for the barcoding charactrization of hematophagous louse flies (Diptera:Hippoboscidae)

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Abstract

The barcoding of life initiative provides a universal molecular tool to distinguish animal species based on the amplification and sequencing of a fragment of the subunit 1 of the cytochrome oxidase (COI) gene. Obtaining good quality DNA for barcoding purposes is a limiting factor, especially in studies conducted on small-sized samples or those requiring the maintenance of the organism as a voucher. In this study, we compared the number of positive amplifications and the quality of the sequences obtained using DNA extraction methods that also differ in their economic costs and time requirements and we applied them for the genetic characterization of louse flies. Four DNA extraction methods were studied: chloroform/isoamyl alcohol, HotShot procedure, Qiagen DNeasy® Tissue and Blood Kit and DNA Kit Maxwell® 16LEV. All the louse flies were morphologically identified as *Ornithophila gestroi* and a single COI-based haplotype was identified. The number of positive amplifications did not differ significantly among DNA extraction procedures. However, the quality of the sequences was significantly lower for the case of the chloroform/isoamyl alcohol procedure with respect to the rest of methods tested here. These results may be useful for the genetic characterization of louse flies, leaving most of the remaining insect as a voucher.

Introduction

Taxonomy currently uses multidisciplinary approaches that combine both morphological and molecular techniques (Bisby *et al.* 2002; Besansky *et al.* 2003; Hajibabaei *et al.* 2007). DNA barcoding provides a useful tool for rapid and accurate identification of species applicable to a wide range of organisms from all fungi, plant, and animal kingdoms (Hebert *et al.* 2003a,b; Hajibabaei *et al.* 2007). In animals, this tool is based on the characterization of a 658bp fragment of a standardized region of the mitochondrial cytochrome c oxidase subunit I (COI) that shows low intraspecific but large interspecific variability (Hebert *et al.* 2003b; Ratnasingham & Hebert, 2007; but see Meier *et al.* 2006; Shearer & Coffroth, 2008).

DNA extraction has been recognized as a critical step for DNA barcode characterization (Ball & Armstrong, 2008) but also may be important in studies using other approaches, including restriction fragment length polymorphism (RFLP) (Möller *et al.* 1992), amplified fragment length polymorphism (AFLP) (Reineke *et al.* 1998), or new generation sequencing (NGS) (Pompanon *et al.* 2012). Current DNA extraction methods can be differentiated into two main groups: commercial kits and standard/traditional methods. Most of these methods are constrained by factors such as the use of hazardous chemicals for human and environmental health (i.e., phenol, chloroform), the need of specialized laboratory equipment (automated DNA extraction), high costs (commercial kits (Petrigh & Fugassa 2013)), and/or time-consumption (Rohland *et al.* 2010). The latter may become an important factor for studies comprising large sample sizes, where automated DNA extraction protocols may significantly reduce manpower requirements (Lee *et al.* 2010). Therefore, it is necessary to evaluate the pros and cons of different DNA extraction procedures to characterize DNA barcodes. Here, we compared the eficacy of four DNA extraction protocols for the genetic characterization of the barcoding region of hematophagous louse flies (Diptera: Hippoboscidae). In spite of the importance of louse flies as blood feeders and potential vectors of different blood parasites (Valkiunas, 2005, Lehane, 2008), precise information regarding the barcode characterization of this insect group is absent for the majority of the species. First, we identified the louse fly species on the basis of distinctive morphological features. Secondly, we used a small leg fragment of these louse flies that were preserved in ethanol during a relatively long period (over six

years) to compare the eficacy of four DNA extraction protocols: two standard protocols, 1) based on the use of chloroform/isoamyl alcohol, and 2) the HotShot (Truett *et al.* 2000), and two commercial kits, 3) a Qiagen kit, and 4) a semiautomatic Maxwell Kit.

Material and methods

We collected 32 louse flies during August and September, 2007 on the islet of Alegranza (10.5 km2, 289 m a.s.l.) in the Canary Islands (27o 37' N, 13o 20' W), Spain. Louse flies were collected from 25-day-old Eleonora's falcon (Falco eleonorae) nestlings. Immediately after collection, each individual louse fly was transferred to a 2 ml Eppendorf tube with ethanol and stored at room temperature until molecular analyses in November, 2013.

Morphological identification of louse flies

Louse flies were identified to species level using available taxonomic keys (Hutson, 1984; Muñoz *et al.* 1993). Nineteen morphological characters were measured in 16 louse flies using a stereo microscope connected to a camera and compared with those previously reported (Muñoz *et al.* 1993).

DNA extraction

We separated the tibia and tarsomere from the middle and hind legs of each louse fly in individual Petri dishes using sterile blades, obtaining a tissue fragment weighing under 0.1 mg. Subsequently, each leg (including tibia and tarsomere) of each louse fly was assigned to one of each four DNA extraction treatments. As a result, 32 segments (eight from the right middle legs, eight from the left middle legs, eight from the right hind legs, and eight from the left hind legs) were assigned for each of the four DNA extraction treatments. According to the chloroform/isoamyl alcohol procedure (Gemmell & Akiyama, 1996), with minor modifications, each sample was introduced into individual tubes containing 300 µl of lysis buffer (100 mM NaCl, 50 mM Tris-HCl pH 8, 50 mM EDTA pH 8, 1% SDS), 5 µl of proteinase K (20 mg/ml), and 10 µl of DDT (1 M) and then kept on a shaker incubating at 55° C overnight. The following day, an equal volume (320 µl) of 5 M LiCl was added to each tube and then each sample was mixed by inversion for 1 min after

adding 630 µl chloroform/isoamyl alcohol (24:1). After shaking the tubes, the samples were centrifuged for 15 min at 13,000 rpm and the supernatant (500 µl) was carefully removed and transferred into a new tube, where 1 ml of absolute ethanol was added to precipitate the DNA overnight at -18° C. The next day, the DNA was recovered by centrifugation at 13,000 rpm for 15 min. The pellet was dried and washed with 70% ethanol, and resuspended in 20 µl of milliQ water. According to the HotShot procedure (Truett *et al.* 2000), each sample was introduced into individual tubes containing 50 µl of lysis solution (25 mM NaOH, 0.2 mM EDTA, pH 8) and then incubated at 95o C for 30 min. After incubation, the solution was put on ice for 5 min and 50 µl of neutralization solution (40 mM Tris-HCl) was added to each sample. Manufacturer specicfiations were used for both commercial kits. These methods allow DNA extraction without organic extractions or ethanol precipitations. Qiagen kit method (DNeasy® Kit Tissue and Blood (Qiagen, Hilden, Germany)), involves enzymatic lysis using proteinase K followed by column purification of DNA using silica-gel-matrix. The semi-automatic Maxwell kit method (Maxwell®16 LEV system Research (Promega, Madison, WI)) involves an enzymatic lysis using proteinase K followed by a purification of DNA using magnetic beads that bind to DNA. The complete process was done in a robot for the simultaneous extraction of 16 samples. For Qiagen and Maxwell kits, DNA samples were diluted in 20µl milliQ water.

The average laboratory time requirement for each DNA extraction method was calculated based on our own measurements. The approximate cost per sample of each procedure was provided by the distributor in Spain (Table 1). Prices could vary depending on the country.

Table 1. Estimation of economic costs (€) of components used in each DNA extraction method and time devoted for the extraction of DNA from 16 samples. Laboratory equipment is not included.

Extraction method	Ease of operation	Cost (per sample)	Time
DNeasy® Kit Tissue and Blood (Qiagen)	Manual	€ 5.71	5 Hours
Maxwell®16 LEV system Research (Promega)	Semi-automatic	€ 3.79	1.25 Hours
HotShot	Manual	< € 1.00	1.5 Hours
Chloroform/isoamyl alcohol	Manual	< € 2.50	6 Hours in 3 days

DNA amplification and sequencing

The primer pair LCO1490 (5'- GGT CAA CAA ATC ATA AAG ATA TTG G -3') and HCO2198 (5'- TAA CTT CAG GGT GAC CAA AAA ATC A -3') (Folmer *et al.* 1994) was used to amplify a 658 bp fragment of the COI gene. PCRs were performed with a final volume of 50 µl containing 0.3 mM each deoxynucleoside triphosphate (dNTP), 0.6 µM of each primer, 2.5 mM MgCl2, 1x PCR bu er (Applied Biosystem, Foster City, CA), 0.6 units of Taq DNA polymerase, and 3 µl of DNA. Following Whiteman *et al.* (2006), PCRs conditions were: an initial denaturation for 4 min (94° C), followed by 35 cycles of 94° C for 1 min. 40 °C for 1 min, and 70° C for 1 min with a final extension at 72° C for 7 min. The presence of amplicons was verified on 1.8% agarose gels.

Sequencing reactions were performed according to the BigDye technology (Applied Biosystems). Positive PCR fragments were resolved in both directions through a 3130xl ABI automated sequencer (Applied Biosystems) using the same primers employed in PCR reactions. Sequences were edited using the SequencherTM v4.9 software (Gene Codes Corp., ©1991-2009, Ann Arbor, MI 48108). Subsequently, Sequencher software was used to quantify the quality value of each sequence obtained by each DNA extraction method after removal of the primer. The quality was measured as the percentage of bases in each sequence with quality scores >20 (see Fazekas *et al.* 2010).

Statistical analyses

Statistical analyses were conducted using General Linear Mixed Models (GLMMs) in SAS (GLIMMIX procedure, SAS Institute Inc., Cary, NC), including a random factor to account for non-independence of samples coming from the same louse fly. First, we fitted a GLMM with binomial error and logistic link function for success (1) or failure (0) of positive amplification of the COI gene as the response variable and extraction method as explanatory factor. Secondly, we fitted a GLMM with normal error and identity link function for the quality of the sequence obtained as the response variable. The DNA extraction method, the sequence direction (forward or reverse), and their interaction were included as fixed factors. In both analyses, louse fly identity was included as a random factor.

Results

All louse flies were identified as *Ornithophila gestroi* on the basis of morphological characters, in particular the patterns of wing venation. In addition, most morphometric measures of louse flies were within the range previously reported for this species (Table 2). A single genetic haplotype of the COI gene was isolated from the 32 louse flies [GenBank accession number: KJ174684]. Tree *O. gestroi* were deposited in the collection of the Museo Nacional de Ciencias Naturales (Madrid, Spain) (accession numbers: MNCN/ADN: 65231-65233).

The DNA extraction method used did not affect significantly the number of positive amplifications ($F_{3, 93} = 0.43$; $P = 0.73$). Amplification was successful for all the samples (n=32) extracted with the Qiagen kit, whereas 29 were successfully amplified using the HotShot procedure and Maxwell kit extraction method and only 26 when using the chloroform/isoamyl alcohol procedure. However, the quality of the sequence obtained was strongly affected by the DNA extraction method ($F_{3, 194} = 8.69$; $P < 0.0001$), while both the sequence direction ($F_{1, 194} = 0.85$; $P = 0.36$) and the interaction between the method and the sequence direction ($F_{3, 194} = 0.44$; $P = 0.72$) had no effect on the sequence quality. The sequence quality obtained when using DNA extracted with the Qiagen kit, the Maxwell kit, and the HotShot procedure was similar (post-hoc tests, $P>0.61$). The quality of the sequences obtained using the chloroform/isoamyl alcohol procedure was significantly lower than that obtained using the other three methods. (post-hoc tests, $P<0.0001$; Fig. 1).

Table 2. Measurements (mm) of different morphological characters of the 16 *Ornithophila gestroi* (W= width; L= length).

Structure	Mean (SD)	Range
Body length	7.94 (1.02)	6.69-9.80
Wing length	6.62 (0.43)	5.76-7.13
Antennae (W)	0.29 (0.03)	0.26-0.34
Lunula (L)	0.32 (0.08)	0.47-0.23
Lunula (W)	0.68 (0.07)	0.54-0.79
Internal orbital width (medium vertex level)	0.19 (0.02)	0.15-0.20
Eye (L)	0.88 (0.08)	0.73-0.97
Eye (W)	0.51 (0.07)	0.38-0.61
Head (L)	1.46 (0.29)	1.34-1.90
Head (W)	2.03 (0.09)	1.88-2.17
Postvertex (L)	0.31 (0.05)	0.23-0.40
Postvertex (W)	0.88 (0.09)	0.77-1.05
Mediovertex (L)	0.52 (0.1)	0.36-0.62
Mediovertex (W)	0.55 (0.05)	0.48-0.63
Prescutum (L)	0.95 (0.11)	0.79-1.12
Scutellum (L)	0.63 (0.07)	0.51-0.72
Scutellum (W)	1.38 (0.18)	1.07-167
Palpi lenght	0.32 (0.1)	0.16-0.43
Minimal distance between ocular margins	0.94 (0.06)	0.84-1.01

Discussion

Genetic characterization of louse flies

Ornithophila gestroi, the species genetically characterized here for the first time, parasitizes different raptor species belonging to the genus Falco, that includes species like the Common Kestrel (*Falco tinnunculus*), the Lesser Kestrel (*Falco naumanni*), and the Eleonora's Falcon (Gil Collado, 1932; Walter 1979,;Beaucournu *et al.* 1985; Gangoso *et al.* 2010), thus representing an important piece for studies on host-pathogen interactions on this avian group. Our results showed the presence of a single genetic haplotype in the louse fly population studied in the Canary Islands. This pattern of low variability at this gene had been previously reported in the louse fly *Trichobius major* (Wilson *et al.* 2007). We cannot

discriminate whether this lack of variation is due to a generally low divergence at the COI gene, the fact that samples were obtained from a single island, or to demographic constraints associated with the geographic isolation of the studied population (e.g., Dasmahapatra & Mallet, 2006). Further studies on the genetic diversity of this species, considering samples from different localities, would be necessary to clarify this issue.

Efficacy of DNA extraction methods

By comparing four different DNA extraction procedures, we found that there were no significant differences in the number of amplifications obtained. However, the quality of the sequences was strongly affected by the method used, with the chloroform/isoamyl alcohol procedure resulting in significantly lower sequence qualities than the other three methods. By using the Qiagen kit, we successfully amplified the 658 bp fragments of all louse flies with high sequence quality. These results are in accordance with previous studies comparing DNA extraction procedures from samples with poorly preserved or degraded DNA (Yang *et al.* 1996; Martínez-de la Puente *et al.* 2013). These results might be especially useful for studies on valuable specimens held in museums, as only a small fragment of tissue was necessary for barcoding while retaining the rest of the specimen as a voucher. However, this procedure is the most expensive of the four methods compared here, which probably may hinder its widespread use (Table 1). To reduce the overall costs of DNA extractions, cleaning methods could be employed to remove any remaining DNA from silica-gel-columns used (Siddappa *et al.* 2007), although this could result in traces of contamination (Fogel & McNally, 2000).

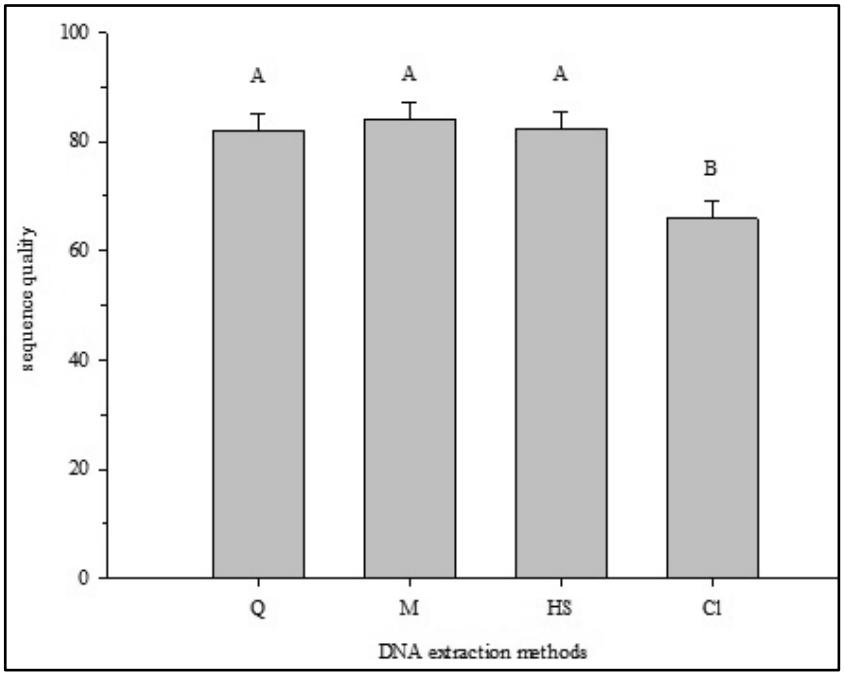

Fig 1. Percentage (± SE) of sequence quality from DNA samples obtained with four different extraction methods (Q= DNeasy® Kit Tissue and Blood (Qiagen); M= Maxwell®16 LEV system Research (Promega); HS= HotShot; Cl= Chloroform/isoamyl alcohol). Dissimilar letters over bars represent significant differences in sequence quality.

Furthermore, we found that the semi-automatic Maxwell kit presented a similar efficacy than the Qiagen kit in terms of the sequence quality, although the amplification success was slightly, but not significantly, lower. These results support those previously obtained by Khokhar *et al.* (2012), who reported that the Maxwell kit is suitable for the extraction of small-size DNA fragments and has the advantage that it requires a limited sample handling (Silva *et al.* 2013). The Hotshot procedure presented similar results to those obtained with the Maxwell kit. Previous studies have already demonstrated the utility of the Hotshot procedure for DNA barcoding using complete individuals (Montero-Pau *et al.* 2008; Lassaad *et al.* 2013). Our results confirmed that the Hotshot procedure yields enough DNA of high quality for barcoding even when using very small quantities of tissue, consequently retaining most of the individual as a voucher.

Finally, we obtained the lowest efficacy, in terms of sequence quality but not in terms of amplification success, using the chloroform/isoamyl alcohol method. This result was unexpected because this method is considered one of the best to obtain DNA of high quality and yield and has been used in studies on barcoding characterization of insects (Gilbert *et al.* 2007). However, the lower performance could be due to the handling of the extremely small samples in our study, which may result in DNA loss and degradation through the DNA extraction process that involves several steps transferring the supernatant from one tube to another. In this respect, this method may be considered useful in those studies requiring organism identification to the species level, where it is not necessary to obtain a complete barcoding sequence (Vesterinen *et al.* 2013).

In conclusion, the commercial Qiagen kit was the most suitable method of DNA extraction of the four tested here. Additionally, the Maxwell method (due to its reduced manpower requirements) and the Hotshot procedure (due to their lower cost) provided similar performance but at significantly lower economic costs. Thee usefulness of the chloroform/isoamyl alcohol method for the characterization of louse fly barcodes is poorly supported by our results.

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Abstract

Avian *Plasmodium* and malaria-like parasites of the genus *Haemoproteus*, are widespread vector-borne parasites commonly found infecting birds. These parasites impose deleterious effects on their vertebrate hosts compromising their survival. While the interaction between these parasites and their vertebrate hosts has received much attention, the study of those factors determining the consequences of parasite infections in the insect vectors has been traditionally neglected. In recent years, factors including the host's parasite load and the mosquito's nutritional status and microbiota have been recorded to play a key role in modulating the impact of parasites on mosquito longevity. Here, we provide a critical review of these evidences to identify gaps in current knowledge and propose future research directions. Further experimental studies are needed to reveal the impact of avian malaria parasites in mosquitoes using realistic conditions found in wild parasite-mosquito assemblages.

Introduction

Avian haemosporidians (phylum: Apicomplexa) are blood parasites infecting birds that are transmitted by insect vectors (Valkiūnas, 2005). Mosquitoes, especially those of the genus Culex, are the main vectors of the avian malaria parasites of the genus *Plasmodium*, although other genera including Anopheles, Aedes and Lutzia may also be involved in their transmission (Santiago-Alarcon *et al*. 2012). In addition to *Plasmodium* parasites, birds are commonly infected by the related malaria-like parasite *Haemoproteus*. Both parasite genera have a similar life cycle but differ in the vectors involved in their transmission, where sexual reproduction of the parasite occurs. Contrary to *Plasmodium* parasites, *Haemoproteus* of the subgenus *Parahaemoproteus* are transmitted by biting midges *Culicoides* (Ceratopogoniidae) while louse flies act as vectors for the subgenus *Haemoproteus*. After an insect vector bites an infected host, parasite gametocytes develop into gametes in the insect midgut and fuse as zygotes to form ookinetes. Subsequently, parasites penetrate into the insect midgut wall where ookinetes develop into oocytes to form sporozoites, the infective form of the parasites. Parasite sporozoites invade the salivary gland of the insect vector where they accumulate until their inoculation to a new host in the following blood meal. Parasite development in the insect vectors usually takes from 8 to 22 days, although *Haemoproteus* sporozoites have been found in the salivary glands of *Culicoides* at 5 dpe (Valkiūnas *et al.* 2002, Valkiūnas, 2005).

By definition, parasites reduce the fitness of their hosts (Poulin, 2011). However, evolutionary theory predicts differential parasite virulence (i.e. the damage done to the host) according to the mode of parasite transmission (Ewald 2004). In vector-borne parasites, virulence may be different in the vertebrate host and the insect vector (Ferguson *et al*. 2003). In fact, vectors may also suffer an important cost of infection in terms of fecundity and survival (Ferguson & Read, 2002), although the impact of avian malaria and malaria-like parasites in the longevity of their insect vectors remains an open question.

Current knowledge

During the last few years, different studies have provided evidence of the costs of avian malaria and malaria-like infections in their insect vectors, including studies on the interaction between *Plasmodium* and mosquitoes (Zélé *et al*. 2012; Lalubin *et al*. 2014) as

well as *Haemoproteus* infecting biting midges (Liutkevičius 2000; Valkiunas & Iezhova 2004; Bukauskaitė *et al*. 2016) and louse flies (Waite *et al*. 2012). However, contradictory results have been frequently reported, with non-significant effects of parasite infections on mosquito longevity (Delhaye *et al.* 2016; Pigeault & Villa 2018) or, even, infected mosquitoes showing an increased lifespan when compared to control-uninfected ones (Vézilier *et al*. 2012). Among other factors, discrepancies between studies could be due to three major factors that have been identified in these studies, including the parasite load of the donor avian host, the vector's nutritional status and the gut microbiota of the insect vectors.

Parasite load in the vertebrate host

Gametocytaemia in birds, defined as the proportion of red blood cells (RBC) infected by gametocytes, is considered a major determinant of the success of malaria parasite development in the vector (Cornet *et al*. 2014). However, an elevated gametocitemia (or parasitaemia) may also increase vector mortality (Ferguson & Read, 2002; Lalubin *et al*. 2014), which becomes evident soon after exposure to infection (i.e. days) (Liutkevičius, 2000). In extreme cases, where insect vectors feed on blood from highly infected birds (5.2% gametocytaemia), mortality was 98% only twelve hours after parasite ingestion (Bukauskaitė *et al*. 2016). The damage produced by parasites in the insect midgut and the immunological costly responses against infections, may explain the high mortality rate found in vectors after feeding on highly parasitized birds (Han *et al*. 2000; Dimopoulos *et al.* 2001) These factors may also explain the pathogenic effect of avian malaria parasites in non-competent insects, as may be the case of *Ochlerotatus cantans* mosquitoes fed on birds with heavy infections by *Haemoproteus* (Vakiūnas *et al*. 2014).

The vector's nutritional status

Host immune responses to fight off parasite infections are energetically costly with nutrition, among other factors, representing a main factor modulating these responses (Sheldon & Verhulst, 1996). Much research has been done in order to explain how the host's nutritional status affects parasitic infections; however, the role of nutritional stress modulating the cost of avian malaria infections in mosquitoes has been poorly studied. In

their seminar paper, Ferguson and Read, (2002) proposed the potential importance of controlling diet on studies on mosquito survival because infected mosquitoes use eight times as much glucose than un-infected mosquitoes (Hurd *et al.* 1995). However, the actual relevance of resource consumption on the mosquito's survival has not traditionally been quantified. The best evidence for the role of the mosquito's nutritional resources on the impact of avian malaria on mosquito longevity derived from the study by Lalubin *et al*. (2014). Authors from this study assigned mosquitoes to two different treatments, either fed with a 2% or a 6% glucose solution diet. A higher mortality rate of naturally *Plasmodium*-infected mosquitoes was only found for mosquitoes feeding on the low-quality diet. Under laboratory conditions, mosquitoes are supplied with carbohydrates from different sources such as fruit juices, honey or 3% to 20% sucrose solutions (Imam *et al*. 2014). Among these, in studies on the effects of avian malaria parasites on mosquitoes, insects are usually supplemented ad libitum with 10% glucose solutions (e.g. Zélé *et al*. 2012), which could partially obscure the deleterious effects of *Plasmodium* infections on mosquito survival (Ferguson *et al.* 2003).

Under natural conditions, mosquitoes from areas with absence of primary natural sugar sources showed reduced longevity when compared with those from an area where flowering Acacia raddiana trees were present (Gu *et al*. 2011). However, under laboratory conditions, mosquitoes fed with 6% sucrose solution showed a lower survival rate than mosquitoes that were fed on plants (Manda *et al*. 2007). In this respect, current studies on the impact of avian malaria parasites on mosquito longevity are likely obscuring the deleterious effects of *Plasmodium* infections as the impact of infections may be palliated by the beneficial experimental conditions. Thus, although the environmental availability of nutrients may differ between wild areas, future studies should consider the natural range of resources to understand the real impact of these parasites on vector survival.

Insect microbiota

Mosquito microbiota modulates the development of human malaria parasites in the vectors (Dong *et al*. 2009) through mechanisms that include direct anti-parasite effects of microbiota on the parasites development (Dong *et al*. 2009) or by stimulating the insect's immune system (Kambris *et al*. 2010). Furthermore, mosquito microbiota may affect the

impact of parasite infections on the mosquito's survival. In their study, Gendrin *et al*. (2015) showed that antibiotic treated *Anopheles gambiae* were more susceptible and had reduced survival due to *Plasmodium* infections, suggesting that this effect was due to the removal of microbiota. In a similar way, endosymbiotic bacteria are expected to modulate the interactions between avian malaria parasites and mosquitoes under natural conditions. In particular, Wolbachia bacteria, which is highly prevalent in the gut microbiota community in the avian malaria vector *Cx. pipiens* (80%, n=15, Muturi *et al.* 2017), may affect the development of avian *Plasmodium* (Zélé *et al*. 2014) and the pathogenic effect of parasites on vector longevity (Zélé *et al*. 2012). Support for this possibility derived from Zélé *et al*. (2012) who found that Wolbachia bacteria protect mosquitoes from *Plasmodium*-induced mortality in a study on *Cx. pipiens quinquefasciatus* with different strains of Wolbachia and Wolbachia-free mosquitoes. However, the mechanisms explaining the interference mediated by Wolbachia on avian malaria development are still uncertain, although an upregulation of the mosquito's immune effect or genes associated to the presence of the Wolbachia may modulate the mosquito's response against parasite infections (Moreira *et al.* 2009).

Further studies on the role of the mosquito's microbiota on parasite development and their impact on mosquitoes' survival are necessary, specially focusing on those factors potentially affecting Wolbachia-mosquito-parasite assemblages. Under natural conditions, the mosquito's microbiota may largely differ between insect species and geographic distribution (Muturi *et al*. 2017; Thongsripong, 2018). In addition, a recent study on the avian malaria-like parasite *Leucocytozoon* and their blackfly vectors, revealed that Wolbachia infections may show positive, negative or, even, neutral associations with parasite infections depending on the vector species studied (Woodford et al 2018).

Concluding remarks and future directions

Avian *Plasmodium* and *Haemoproteus* impact the longevity of their vectors, as supported by studies on mosquitoes, biting midges and louse flies (Table 1). However, important discrepancies between studies have been reported. Here, we report evidence for the role of three major factors determining the impact of parasites on insect vectors,

including the parasite load in the vertebrate host and mosquito-related factors (i.e. the nutritional stress, gut microbiota).

Table 1. A selection of studies published on the effects of avian malaria and the malaria-like parasites *Haemoproteus* on vector longevity. Studies are sorted according to the parasite-vector studied and chronologically. NS: non-significant effects of *Plasmodium* infection, - : reduce lifespan of insects; + : increase lifespan of insect.

Vectors	Parasite (lineage)	Effect	Ref
Mosquitoes			
Cx. pipiens	*P. relictum* (SGS1)	+	Vézilier et al. (2012)
Cx. pipiens quinquefasciatus	*P. relictum* (SGS1)	- *	Zélé et al. (2012)
Cx. pipiens	*Plasmodium* spp.	- **	Lalubin et al. (2014)
Cx. pipiens	*P. relictum* (SGS1)	+***	Pigeault et al. (2015)
Cx. pipiens	*P. relictum* (SGS1)	NS	Delhaye et al. (2016)
Cx. pipiens	*P. relictum* (SGS1)	NS	Pigeault and Villa (2018)
Biting midges			
Culicoides impunctatus	*Haemoproteus dolniki* *H. balmorali* *H. tartakovskyi* *H. belopolskyi*	-	Liutkevičius (2000)
C. impunctatus	*H. fringillae* *H. lanii*	-	Valkiūnas & Iezhova (2004)
C. impunctatus	*H. lanii* (hRB1)	-	Bukauskaitė et al. (2016)
Louse flies			
Pseudolynchia canariensis	*Haemoproteus columbae*	-	Waite et al. (2012)

* The negative effects on mosquito survival were especially evident for the case of *Wolbachia*-free mosquitoes. ** The significant negative effect of parasite infection on mosquito longevity was found only on insect supplemented with a low (2%) sugar solution. *** Mosquito longevity was positively associated with bird parasitaemia, although did not with the bird *Plasmodium* infection status.

A number of different avian *Plasmodium* lineages are commonly found harboured by wild mosquitoes (Ferraguti *et al*. 2013). However, most studies conducted until now are restricted to a handful of parasite-vector assemblages, most of them focus on the extensively studied interaction between *Culex pipiens* and the *Plasmodium relictum* lineage SGS1, thus under-representing the diversity of interactions found under natural conditions.

Therefore, further studies using additional parasite species/lineages and vector species are necessary. This is especially important to conduct reliable epidemiological studies as information on the costs of parasite infections on insect vectors is essential to estimate the transmission risk of malarial parasites under natural conditions. In fact, epidemiological models based on the quantification of the basic reproductive ratio (R_0, defined as the number of secondary infections from an infected individual) requires basic data on the longevity of infected mosquitoes (Ross, 1911; Macdonald, 1955), with is currently lacking for most of the wild avian parasite-vector assemblages.

Moreover, it is known that avian *Plasmodium* species differ in their virulence in their vertebrate hosts (Lachish *et al.* 2011), and this could be the case in the insect vectors too. However, Valkiūnas and Iezhova (2004) did not find differences in the mortality rate of *C. impunctatus* infected by three *Haemoproteus* parasites. In addition, although studies conducted until now provide valuable information on the mechanisms underlying the parasite costs' on insect vectors, differences in the magnitude of these impacts may be affected by the use of parasite strains-maintained thought serial passages in birds. This procedure may affect parasite virulence (Ebert, 1998; Mackinnon & Read 2004). For example, for the avian *Plasmodium*, serial passages slightly increased parasitaemia in birds, measured as the proportion of parasite infected RBCs, while gametocitemia remained constant (Pigeault *et al*. 2015). These, together with the differential conditions for vectors that may have been used between studies (i.e. supplemented sugar concentration, temperature, relative humidity) may partially explain discrepancies found in the literature.

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El fin de la carrera estaba a la vuelta de la esquina y, aunque muchos de los amigos ya tenían muy claro a que querían dedicarse, yo aún tenía mis dudas, ¿enseñanza o investigación? Pero un día apareció un cartel en la Facultad que anunciaba la posibilidad de hacer prácticas de empresas en la Estación Biológica de Doñana. Dado que apenas tenía dos o tres asignaturas ese último cuatrimestre, entregué mi mini CV, y a las pocas semanas, ¡sorpresa! una llamada diciéndome que había sido seleccionado. Olé tú!

Miento si digo que no me temblaba todo el cuerpo cuando entré en ese edificio por primera vez. Por entonces no lo sabía, pero empezaba una gran etapa en mi vida. Durante esos 4-5 meses que estuve como alumno colaborador en la EBD conocí a personas increíbles a las que les tengo que agradecer como me recibieron, me trataron y todo lo que enseñaron durante ese tiempo (Gracias Eva, Laura, Mireia, Bego, Michael Jowers, Isa Martín, Quini y Juanele).

Tras acabar las prácticas en la EBD, decidí subir mi CV a la bolsa de empleo, y apenas unos meses más tarde recibo una llamada de un tal Alfredo Valido que, tras una entrevista, decide contratarme como técnico. Paradojas de la vida, volvía a la EBD. Fue casi un año y medio que me sirvió, no solo para coger una gran experiencia en un laboratorio de molecular como es el LEM, sino también para conocer Mogán, su puerto y su vieja al horno, además de conocer a grandes personas. Gracias Alfredo, Pedro, Nestor, Manolo Carrión, Cande, Rocío, JuanMi, Cristina, Gasent, Antonio, María y Mónica por todos los buenos momentos vividos. Gracias a la experiencia vivida con vosotros me di cuenta que algo dentro de mi pedía más caña, esas constantes preguntas que me hacía a mí mismo del porqué de las cosas, me hicieron ver que quería y que estaba preparado para hacer una tesis doctoral.

Pero antes de que empezara la tesis, faltaba un pequeño obstáculo. Había que hacer un máster. Durante mis largos periodos en el LEM, conocí a un nuevo chaval que apareció por la EBD por el 2011 y que de vez en cuando se acercaba por mi poyata para preguntar cómo me iba. Siempre iba con una sonrisa en la boca, siempre tratando súper bien a sus técnicos, así como a los diferentes alumnos en prácticas que estaban con él. Poco a poco fui conociendo con que trabajaba (¡con mosquitos!), y la verdad es que me molaba mucho lo que hacía (¿será porque gran parte de mi vida había vivido en la Puebla del Rio, cuna de los mosquitos por excelencia en

Sevilla? Pudiera ser). Así que le dije que iba a hacer el máster de la UPO y que me gustaría hacer el TFM con ellos. Y así fue como empecé en este maravilloso grupo/familia a los que tanto les debo y de los que no podría estar más agradecido ("LOS FIGUERÓLIDOS").

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Pero en realidad, contigo empezó todo Josué. Tú fuiste el primero que confió en mí y me abriste las puertas para ser parte vuestra. Me has enseñado todo lo que se sobre el mundo de la parasitología y sus vectores, ya sea directa o indirectamente. Me has apoyado en todos esos malos momentos que un doctorando pasa a lo largo de esta etapa (aunque he de decir, que han sido muy pocos). Gracias por todas esas risas en la EBD. Sin duda no tengo palabras que puedan expresar lo que te agradezco por todo cuanto has hecho por mí. ¡Gracias Josué! (Gracias también a Isabel Moreno por tranquilizarlo los días que lo saturaba).

Pero esta tesis estaría incompleta sin la supervisión de una gran mujer. Una mujer que posiblemente tenga más huevos que muchos de los hombres de la famosa serie de Narcos. Pero es todo fachada, como se suele decir, y por dentro es dulce como los donuts de chocolate (¡que por cierto tanto le gustan!). Sin duda era y es un placer sentarme al lado tuya a aprender de estadística en R o JMP. Todo lo que se de estadística es gracias a ti. Me hubiera gustado poder vivir estos últimos meses contigo en directo y no por Skype, aunque me temo que son "cosas del directo". ¡Gracias Laura!

He de reconocer que a veces ha sido "difícil" tener tres supervisores. No siempre pensabais igual, pero sin duda, esta tesis estaría "coja" si alguno de vosotros tres no hubiera estado, pues os complementabais entre vosotros y cada uno es un

pilar de esta tesis. Todo ello, unido a la ayuda de Ramón Soriguer (como decimos los predoctorales del grupo, el "abuelo"). Gracias Ramón por tener siempre palabras de ánimos y por enseñarme de la vida en general (y por esos buenos vinos y quesos de la familia Soriguer). Gracias a los cuatro por haber hecho que siga haciéndome nuevas preguntas constantemente y que no haya perdido nunca las ganas de preguntarme el porqué y el cómo de las cosas. Sé que los cuatro os habéis dejado la piel conmigo, y que muchas veces os he exprimido, por ello también os pido, al mismo tiempo, disculpas y gracias por dejaros "exprimir". Ahora llega un punto y seguido en esta carrera científica, en donde, tarde o temprano, os tendré que dejar (muy a mi pesar, pues nadie que estuviera cuerdo quisiera dejar de estar junto a vosotros) y empezar a volar yo solo (es ley de vida o ley de ciencia, según se quiera llamar). Aun así, estoy seguro que volveremos a coincidir en el camino.

Si los supervisores habéis sido cada uno un pilar de esta tesis (junto con Ramón), el cuarto pilar ha estado compuesto por todas las personas que componen el grupo.

Martina, esa muchacha que siempre está sonriendo y que cuida el karma como nadie. Dispuesta a ayudarme en todo momento (¡eso sí, a 5 pavos!! Jajaja). Estos cuatro años hubieran sido sin duda muy aburridos si tú no hubieras estado aquí. Solo nos quedan pendiente dos cosas por hacer: 1) salir de discotequeo del bueno, 2) ese Ferraguti & Gutiérrez-López. Grazie mille Marti per essere come sei.

Pero también está Alazne, la otra predoctoral del grupo. Una persona de las que quedan pocas en este mundo, pues me costó la misma vida convencerla para que me aceptara invitarla a un café. Esos viajes a por mosquitos a Huelva en la Kangoo en donde los dos nos contábamos todo aquello que nos tenía "alterado" y que nos hacía volver como nuevo, o ese día de ~~vacaciones~~ captura de larvas por Doñana en el que disfrutamos como dos niños chicos, son cosas que no olvidaré jamás de ti. Mil gracias Alazne por todos esos momentos.

Pero este grupo, si se caracteriza por algo es porque es paritario, por lo tanto, también había un hombre más predoctoral, mi gran amigo Jiayue Yan. Compañero de batallas con los mosquitos y gorriones durante el verano de 2014. Un tío con el que no me he podido reír más en mi vida y al cual nunca lo he visto enfadado (salvo cuando hablaba en chino, que parecía que estaba enfadado). Me has enseñado mucho

más de lo que te puedes imaginar. Has sido para mí un ejemplo de superación personal. 谢谢 (xie xie) Jiayue.

Por último, pero no menos importante, aunque haya llegado la última, está Jéssica, la pequeñaja, y es que ella es como esa hermana pequeña que no tuve, esa que, aunque uno esté ahí continuamente "pinchándola", siempre está al otro lado de la mesa para preguntarte que te pasa cuando te ve resoplar delante de la pantalla. Eres un solete Jessica, ¡Gracias!

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Isa y Laura (Gómez), gracias, porque habéis sido mis manos y mis ojos en el laboratorio, sin vosotras esto no hubiera sido posible. ¡Sois dos profesionales como la copa de un pino! Me siento que tengo una deuda con vosotras imposible de devolver. Si algún día soy un investigador con dinero os intentaré contratar (¡si vosotras queréis, claro está!)

Y tú, Alberto, aunque ornitólogo de profesión exterior, eres maestro en el interior. Contigo he aprendido de ornitología lo que no está en los escritos, me has enseñado a observar la naturaleza de forma diferente, sin duda tienes un don y he sido un afortunado al tenerte a mi lado en mi tesis.

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El lugar de trabajo, en concreto los centros de investigación, son uno de los lugares donde las relaciones sociales se hacen más intensas, dado el gran tiempo que se pasa en su interior. La EBD, es uno de esos centros, pero tiene algo especial. Algo, que hace que se pueda convertir en algo como tu casa. Un compañero me contó que hay tres hechos que indican que una persona está convirtiendo su lugar de trabajo en

su propia casa. La primera, es traerse el cargador del móvil (dada la duración de los móviles de hoy en día, eso sucede el primer día), la segunda es el cepillo de dientes (que tampoco es que tardara muchos más días en llevarlo al trabajo), y la tercera es que al llegar a casa intentes abrir la puerta con las llaves del despacho. Esto último, en mi caso no se ha dado, dado que a la EBD se entra mediante el uso de tarjeta, pero este hecho podría ser fácilmente sustituido por el de intentar pagar el autobús con la tarjeta de la EBD en vez del bonobús y, tras pasar la tarjeta dos veces o más, ver como el conductor te mira con cara de estar llamándote "estúpido". Dado que los tres hechos se han cumplido, y de forma reiterada, puedo decir, y estoy orgulloso de ello, que la EBD se ha convertido en mi casa. Y, ¡ole tú! ¡Vaya casa! En esta casa no pesan las horas, y no pesan porque cuando empiezan a pesar aparece por la puerta Jesús Gómez para decirte cualquier tontería, pedirte un descansito o que vayamos a ver a Óscar Gordo (un señor amigo de los de verdad para toda la vida) y hablemos del sistema de contratación de las Universidades. Que resulta que Jesús no aparece, pues ya voy yo a su despacho, y allí te encuentras también con Burraco, y si estamos los tres, (Jesús, Burraco y uno mismo), pues imagínate la conversación Random que puede salir de ahí. Eso sin contar que no se sume Fran (el futuro marido de Martina) o Lucas, porque entonces ya, como se suele decir "apaga y vámonos".

Pero cuando no eran descansos, eran las mesas redondas, organizadas por Vane y Edu y posteriormente por Víctor y Vanina. Mesas redondas que hacían que desconectaras y tuvieras otra visión de las cosas con las experiencias de los investigadores más seniors o personas del exterior. Chapó por los organizadores de esas mesas redondas.

Pero sin duda, los mejores momentos son esos almuerzos donde nos reunimos todos y que se pueden alargar lo que uno desee (según Noa, una hora mínima, y nada de hablar de trabajo), almuerzos en donde puedes probar variedad de riquísimos platos elaborados por Alazne, Martina, Irene o Edu, o quedarte embobado contando las historias de J y Edu al estilo chiquito. Almuerzos que no son los mismos si no escuchas esas contestaciones tan chulescas entre los madrileños (Jesús y Sara principalmente) con sus "ej que", o esos contraplanes de Rosita, pidiendo que comamos fuera ¡que hace solecito! También he de decir que os debo la vida a Jesús, Burraco, Noa, Edu, J, Fran, Martina, Alazne, Laura (Gómez) y no

recuerdo quién más estaba ese día, pero gracias a todos por como actuasteis. A toro pasado todo son risas, pero sé que ese almuerzo lo pasasteis mal.

Quizás no os nombre a todos, pero eso no significa que no os tenga en mente, pues al fin y al cabo lo que quiero manifestar con este último párrafo es el mayor de los agradecimientos a todos los doctorandos de la EBD que hace que nos sentamos como en casa. GRACIAS DOCTORANDOS

Pero no todo es la EBD, en la universidad coincidimos las mejores personas que existían en Sevilla en aquellos momentos (cada uno de su padre y de su madre, y con sus propias taritas), fundándose lo que más tarde se llamó "la Gigapandi" (no os nombro uno a uno porque seguro que me olvido de Ezequiel, ¡jajaja!). Gracias chic@s por los buenos momentos que vivimos cada vez que nos juntamos que hacen que el tiempo se pare. ¡Sois únicos!

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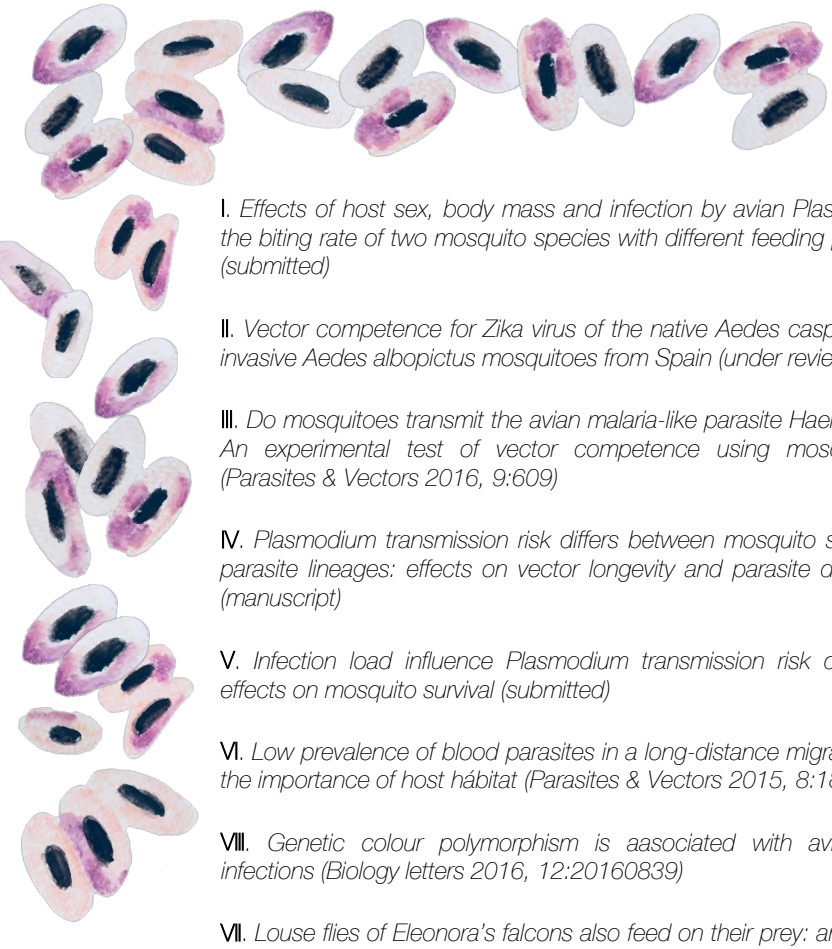
Ahora, sí que sí, el último párrafo de la tesis. Con él parece que ya llega el final, pero como dijo Fred Brooks "las tesis no se acaban; se abandonan." Y es que en realidad esto no es más que el principio, (sí, leíste bien), el principio de mi carrera como investigador. Y, ¿qué es lo que está por venir? Pues, del futuro lo único que sé

es que nunca caminaré solo, porqué tengo claro que siempre tendré a gente importante a mi lado, Merche, Papá, Mamá, Hermano, Amigos.

"*Para conocer el camino que aún te falta por recorrer,*
pregúntale a los que ya vienen de vuelta"

-proverbio chino-



I. *Effects of host sex, body mass and infection by avian Plasmodium on the biting rate of two mosquito species with different feeding preferences (submitted)*

II. *Vector competence for Zika virus of the native Aedes caspius and the invasive Aedes albopictus mosquitoes from Spain (under review)*

III. *Do mosquitoes transmit the avian malaria-like parasite Haemoproteus? An experimental test of vector competence using mosquito saliva (Parasites & Vectors 2016, 9:609)*

IV. *Plasmodium transmission risk differs between mosquito species and parasite lineages: effects on vector longevity and parasite development (manuscript)*

V. *Infection load influence Plasmodium transmission risk due to their effects on mosquito survival (submitted)*

VI. *Low prevalence of blood parasites in a long-distance migratory raptor: the importance of host hábitat (Parasites & Vectors 2015, 8:189)*

VIII. *Genetic colour polymorphism is aasociated with avian malaria infections (Biology letters 2016, 12:20160839)*

VII. *Louse flies of Eleonora's falcons also feed on their prey: an alternative transmission pathway for blood parasites or an evolutionary "cul de sac"? (manuscript)*

Annex I. *Comparison of manual and semi-automatic DNA extraction protocols for the barcoding characterization of hematophagous louse flies (Diptera:Hippoboscidae) (Journal of Vector Ecology, 2015, 40: 11-15)*

Annex II. *Do avian malaria parasites reduce vector longevity? On the effects of parasite load, mosquito nutritional status and gut microbiota (manuscript)*

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