Parásitos maláricos y sus consecuencias en el comportamiento y la eficacia biológica de Passeriformes

Tesis doctoral

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Departamento de Anatomía, Biología Celular y Zoología

UNIVERSIDAD DE EXTREMADURA

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Conforme el director de la Tesis

Fdo: Dr. Alfonso Marzal Reynolds

A mis padres y hermanas.
A Kamylo

CONTENTS

This thesis is base on the following papers which are referred to by their Roman numerals:

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Parásitos maláricos y sus consecuencias en el comportamiento y la eficacia biológica de Passeriformes

INTRODUCTION

Importance of parasites

The human population living nowadays in the planet is approximately 7,000 millions of people (George 2009). This figure is increasing each minute with the number of births taking place in the whole world (330 new babies / minute) (Cohen 2003). However, these data become insignificant if we compare them with the number of parasite living in the Earth. Every living organism, including humans, has at least one parasite that lives inside or on it, and many have far more. For instance, some frog species may harbor a dozen species of parasites, including nematodes in their ears, filarial worms in their veins, and flukes in their kidneys (Lees 1962). Additionally, a bird can carry on its feathers more than 30 different species of mites (Jovani and Blanco 2000). Concerning humans, one third of the population is infected by the bacteria causing tuberculosis (*Mycobacterium tuberculosis*) (Álvarez et al. 2009) and around 300-500 million of people get the infection by *Plasmodium* spp. each year (Gardner et al. 2002). Taking into account the broad presence of parasites in the Earth, it is logical to think that humans, despite having colonised the whole word, are not the most widespread organism in the planet. In fact, the most ubiquitous organisms all over the world are parasites, as they do not infect only humans but also the rest of animal species, plants, fungus, bacteria and viruses (Bush et al. 2001).

Similarly to the rest of vertebrates, birds coexist with a great number of parasites (Clayton and Moore 1997). Parasites may exert a strong selection pressure in birds, determining life histories of their avian hosts. For example, the Hamilton and Zuk hypothesis posits that sexual ornaments are indicators of parasite and disease resistance (Hamilton and Zuk 1982) demonstrating the role of parasites provoke in the mate choice of birds. Therefore, due to the intense selection that parasites may exert on their avian hosts, the relationships found between birds and their parasites are an increased source of knowledge for evolutionary science (Loye and Zuk 1991). As a consequence, the number of studies exploring this host-parasite relationship in birds has increased in the last twenty years (Figure 1).

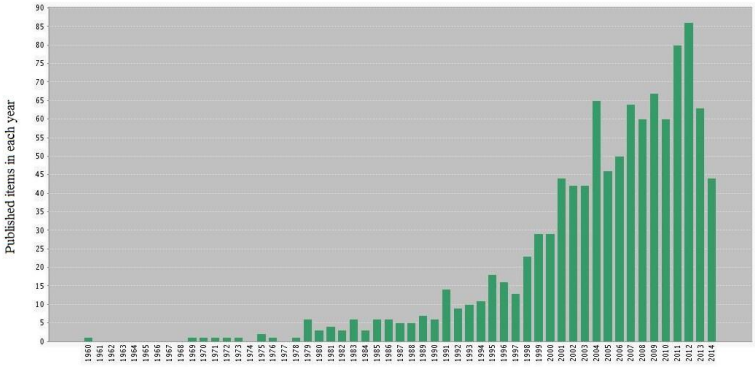


Figure 1. Increase in number of articles published within the framework of "avian host-parasite" over the period 1960 – 2014. The figure is based on a literature search in the ISI-Web of Science (Thomson, December 2014).

Avian malaria and related haemosporidian are one of the most studied bird parasites (e.g. Garamszegi 2011; Howe et al. 2012; Podmokła et al. 2014). They are diverse and abundant organisms infecting several hundred species of birds in almost all continents (Van Riper III et al. 1986; Valkiūnas 2005). As a consequence of the infection, haemosporidian parasites may provoke detrimental effects on their avian hosts affecting survival (Van Riper III et al. 1986; Valkiūnas

2005), diminishing host body condition (Merino et al. 2000; Valkiūnas et al. 2006) and decreasing reproductive success (Merilä and Andersson 1999; Asghar et al. 2011). Within the suborder *Haemosporina*, the genera *Plasmodium*, *Haemoproteus* and *Leucocytozoon* are the most abundant avian parasites. Their life cycle is quite complex, involving both sexual and asexual stages. Their transmission from infected to uninfected hosts usually involves the presence of arthropod vectors. These vectors are different among the three genera: blood-sucking mosquitoes are the main vectors of avian *Plasmodium*, whereas biting midges and hippoboscid flies are the vectors of *Haemoproteus* and simuliid flies transmit *Leucocytozoon* (Valkiūnas 2005).

Despite the large number of researches focused on haemosporidians parasites in recent years, there is still limited knowledge on many different issues concerning host-parasite interaction. i) For example, several studies have shown a relationship between parasite infection and the behaviour of their hosts (see review in Moore 2002), but the link between haemosporidian parasites and the escape behaviour of their avian hosts remains unknown. ii) Moreover, no study has previously experimentally tested the effect that malaria parasite may provoke in the escape behaviour of their avian host. iii) Furthermore, despite the ability of *Plasmodium relictum* lineages to be present in almost all continents (Snow et al. 2005; Palinauskas et al. 2007) and their invasiveness in many island avifauna (Van Riper III et al. 1986; Howe et al. 2012), no study has shown the presence of the malaria lineage *P. relictum* SGS1 infecting birds in mainland Americas. Other issued needing a deeper analysis is the identification and detection of essential genes involved in the life cycle of avian malaria. Although some of these essential genes have been analyzed (Escalante and Ayala 1994), there is still a significant number of crucial genes that have not been detected. iv) For instance, the chitinase gene, a critical gene allowing to malaria parasite trespassing the midgut of the mosquito (Tsai et al. 2001; Li et al. 2004), has not been identify in one of the most widespread and harmful avian malaria parasite, *P. relictum*. v) Similarly, the MSP1 gene, required for allowing the entrance of the malaria parasite to the red blood cell (Gerold et al. 1996) has been scarcely used for epidemiological studies of birds (Hellgren et al. 2014). vi) Finally, the number of studies that have correlated the effects of haemosporidian infection on feather grow rate are still limited, and they are mainly focus on one single species (e.g. Marzal et al. 2013a, Marzal et al. 2013b).

These issues constitute the main research core of this thesis. Next each of these aims of studies will be briefly introduced.

Blood parasites and bird escape behaviour

The first means of avoidance of predation is fleeing, as reflected by flight initiation distance in birds when a predator is approaching (Hediger 1934; Blumstein et al. 2006). But once a bird is already captured by a predator, a suite of different behaviours are usually performed by the birds in order to escape from the hunter (Møller et al. 2011). Among others, escape behaviour includes the intensity with which a captured individual wriggles to escape, biting, loosing feathers, displaying alarm and distress calls, and

maintaining a tonic immobility as a behaviour akin to feigning death (Møller et al. 2006).

Parasites may affect different aspects of host behaviour in ways to increase their own fitness (Moore 2002; Schmid-Hempel 2011). In this sense, the *behavioural manipulation hypothesis* posits that manipulation of host behaviour by parasites must confer fitness benefits to the parasite, usually by achieving higher transmission success than conspecifics that are unable to modify their host behaviour (Lefèvre et al. 2008; Poulin 2010). For instance, it has been shown that *Plasmodium gallinaceum* is able to increase the biting rate of its mosquito vector *Aedes aegypti*, leading to an increase in its transmission success (Koella et al. 2002). Also, Cornet et al. (2013) experimentally demonstrated that infected birds attracted a significantly higher number of vectors than uninfected ones. Results from these studies suggest that malaria parasites may manipulate the behaviour of their invertebrate hosts (vectors) to increase their own transmission. However, whether haemosporidian parasites may influence behaviour of their bird hosts is largely unknown (Dunn et al. 2011; Garcia-Longoria et al. 2014). With this aim, in chapter I the relationship between haemosporidian infection and the escape behaviour of different species of birds will be explored. In addition, the effects of malaria infection on the escape behaviour of house sparrows will be experimentally studied in chapter II.

***Plasmodium relictum* SGS1 as invasive malaria species in South America**

In last centuries, many species have been successfully introduced and become invaders in many parts of the world (Hatcher and Dunn 2011). Malaria and related haemosporidian parasites have also been introduced in different areas of the world. *Plasmodium relictum* is among the most invasive species of avian malaria, being responsible of mass mortality and even extinctions of many native bird species worldwide after its introduction outside its native range (Van Riper III et al. 1986; Valkiūnas 2005). As a consequence, the International Union for Conservation of Nature (IUCN) classifies avian malaria *P. relictum* to be among the 100 of the world's worst invasive alien species (Lowe et al. 2000).

The application of modern molecular methodologies based on DNA sequencing has allowed the identification of different DNA lineages linked to different morphospecies of avian haemosporidians (Bensch et al. 2000; Bensch et al. 2009). For example, based on partial sequences of the cytochrome b gene, GRW4 and SGS1 are two lineages of morphospecies *Plasmodium relictum* with different distribution areas. While *P. relictum* lineage SGS1 is a widespread and actively transmitted parasite lineage in Europe, Africa and Asia (Palinauskas et al. 2007), the geographical range of *P. relictum* lineage GRW4 includes New Zealand, Africa, Asia and the Americas (Beadell et al. 2006; Marzal et al. 2011). Both sister parasites lineages might easily switch to new hosts and invade new areas (Beadell et al. 2006; Hellgren et al. 2009). Therefore, it becomes crucial to identify the geographical distribution of *P. relictum*

lineages and their infection prevalence in birds. With this aim, the presence of SGS1 in Neotropical birds from two different areas of Peru will be analyzed in chapter III.

Identification and characterization of chitinase gene in Plasmodium relictum

Arthropod vectors develop a protective peritrophic membrane (PM) around their midgut after each blood meal (Sieber et al. 1991). This membrane may act as a barrier blocking the penetration of blood parasites and thus not allowing them to spread to other organs (Ghosh et al. 2000). Hence, malaria parasites should overcome this barrier (PM) in order to complete their life cycle. The mechanism that allows malaria parasites to go through the PM of their vectors is well known. For example, *Plasmodium* ookinete has been shown to be able to cross this barrier by secreting a chitinase allowing them to trespass the PM (Tsai et al. 2001; Kadota et al. 2004; Li et al. 2004). Therefore, the secretion of chitinase is an essential step in the completion of the life cycle of malaria parasites.

The diversity and structure of chitinase gene has been well studies in rodent, primate and human malaria (Li et al. 2005). *Plasmodium gallinaceum* has been the primary model for studies related with chitinase function in avian malaria (Sieber et al. 1991; Shahabuddin et al. 1993). However, this species mainly affect poultry species, and it is not the most common malaria parasite in wild birds. *Plasmodium relictum* is the most widespread and harmful avian malaria parasite (Bensch et al. 2009). However, no study has searched for the presence and identification of the structure of chitinase gene in this malaria species. With this aim, the presence, structure and genetic variability of the gene encoding for chitinase will be analyzed in chapter IV in the two most widespread lineages of *P. relictum* (SGS1 and GRW4).

Detection of MSP1 gene in house martins infected by P. relictum

The merozoite surface protein 1 (MSP1) is essential for malaria parasite in the erythrocyte invasion process. During erythrocytic schizogony (merogony), MPS1 is anchored to the parasites' cell membrane allowing the entrance of the malaria parasite to the red blood cell (Gerold et al. 1996). Because its high variability this gene has been used to infer the structure and phylogeograhpy in populations of primates (Noranate et al. 2009; Kang et al. 2012; Pacheco et al. 2012; Tanabe et al. 2013). Similarly, Hellgren et al. (2013) identified the avian MSP1 gene, allowing futures studies to detect this gene in birds infected with *P. relictum* lineages (SGS1 and GRW4).

The global distribution of MSP1 allele in *Plasmodium relictum* lineages has been recently shown (Hellgren et al. 2015). This finding allows future studies to explore the genetic variation of these lineages among different populations of birds. Because migratory movements in birds may facilitate the spread of blood parasites (Santiago-Alarcon et al. 2013), migratory birds can carry lineages of *Plasmodium* parasite to new areas facilitating the spread of the disease. The house martin (*Delichon urbica*) is a well-know migratory passerine. This species migrates from Africa to Europe every spring to breed. Once bred is

complete, adult house martins and new-born individuals travel back to their African wintering quarters (Cramp and Perrins 1994). *Plasmodium relictum* SGS1 and GRW4 are widespread parasites. SGS1 can be found in some African and European resident bird species (Hellgren et al. 2009). In addition, GRW4 has been detected in some New Zealand, Africa, Asia and America resident species (Beadell et al. 2006; Marzal et al. 2011). Thus, GRW4 and SGS1 can be found in almost all continents. Despite previous studies have shown that house martins may be infected with SGS1 (Marzal et al. 2008), the area where this species catch the infection remains unknown. Because house martins are exposed to both African and European parasite fauna, the infection by *P. relictum* could take place in one, another or both areas. In chapter V it will be analysed the diversity of MSP1 in order to shed light on the malaria infection of the house martins and thus determining where do house martins get the infection by *P. relictum*.

Feather grow rate and malaria parasites

Feathers are essential for flight, but they can also provide streamlining, insulation, camouflage, waterproofing and may act as sexual traits involved in female mate choice (Proctor and Lynch 1994). Daily activities such as rubbing, preening and dust bathing all subject feathers to physical abrasion that causes wear in the plumage (Butler and Johnson 2004). As damage accumulates, the functional properties of feathers are compromised, and hence birds must replace them in order to maintain plumage functions in a process named moult. During moult period birds may suffer from a higher exposition to predators (Lind 2001), a reduction on its flight performance (Williams and Swaddle 2003) and a decrease in thermoregulation (Ginn and Melville 1983). Hence, natural selection should favour the regeneration of feathers as rapidly as possible. However, moult requires a significant investment of energy such as changes in nutritional demands (Klaassen 1995; Murphy 1996) or reallocation of resources among organs and functions (Murphy and Taruscio 1995; Murphy 1996; Nava et al. 2001). Because resources are usually limited, the rate of feather growth during moult can be affected by factors such as body condition, nutritional status, physiological stress and diseases (DesRochers et al. 2009; Moreno-Rueda 2010; Vágási et al. 2012).

Although some studies have shown that blood parasite infection are linked with moulting (Morales et al. 2007; Tarello 2007, but also see Allander and Sundberg 1997), the negative correlation between malaria parasites infection and feather growth rate has only been shown in a migratory species (Marzal et al. 2013b; Marzal et al. 2013a). In chapter VI it will be explored whether haemosporidian infection may affect feather growth rate in the resident house sparrow *Passer domesticus*. Moreover, this negative relationship will be also experimentally tested in captivity to assess whether malaria parasites decrease feather growth rate of their hosts by breaking up any correlation with potentially confounding variables.

AIM OF THE THESIS

The general goal of this thesis was to obtain a better understanding of the consequences of haemosporidian parasites infection in birds, combining different sources such as observational, experimental and molecular data. Although along this thesis malaria parasites (*Plasmodium*) was the main focus, others haemosporidians genera (*Haemoproteus* and *Leucocytozoon*) were also analyzed.

Specifically, six aspects of host-parasites interactions were analyzed. The main objectives of the thesis can be listed as follows:

1. To analyze the relationship between haemosporidians parasite prevalence and escape behaviour in different species of birds (chapter I).

2. To experimentally test whether malaria parasite may provoke any alteration in the escape behaviour of house sparrows (chapter II).

3. To examine the prevalence and genetic characterization of avian malaria and related haemosporidian parasites in Neotropical birds from two different regions of Peru (chapter III).

4. To identify the gene encoding for the chitinase in one of the most widespread and harmful avian malaria parasite (*Plasmodium relictum*) (chapter IV).

5. To detect the merozoite surface proteine 1 (MSP1) in *Plasmodium relictum* (SGS1 and GRW4 lineages) infecting house martins with the aim to identify the potential areas of transmission (chapter V).

6. To explore whether malaria parasites may affect feather growth rate in the resident house sparrow under natural and experimental conditions (chapter VI).

MATERIAL AND METHODS

Study species

In Chapter I, an extended data set was used in order to estimate the relationship between haemosporidians parasites and escape behaviour. The analyses on the relationships between the escape behaviour of the hosts and the blood parasite infection were carried out in 85 Passeriform species.

House sparrows are one of the most widespread and ubiquitous bird species (Summers-Smith 1988). Moreover, this species shows high prevalence and diversity of haemosporidians parasites, being one of the most parasitized passerine species (Marzal et al. 2011; MalAvi 11-09-2014; Bensch et al. 2009). Hence, a considerable number of studies have chosen this bird species as a model for blood parasites studies

Lima et al. 2010; Loiseau et al. 2011; Coon and Martin 2014). Because the distribution and the high parasite prevalence shown by this species, house sparrows were used to explore the relationship between *Plasmodium* prevalence and escape behaviour (chapter II) and to test the effect of malaria parasite in both feather grow rate and

condition assessments (chapter VI).

Figure 2: A male house sparrow in its natural habitat.

The house martin is a migratory species from the Hirundinidae family (Cramp and Perrins 1994). House martins show a high fidelity to their area of hatching and nesting. This feature makes them the perfect avian model in order to determine the effect of malaria parasite in their range of survival among time. Because of the aforementioned characteristics, this model species was used to explore the infection dynamics between Europe and Africa (chapter III).

Figure 3: House martins feeding in their natural habitat.

Areas of study

The information on escape behavior (chapter I) was derived from a previous study of extensive capture of birds during 2008–2012 in Denmark, Sweden, and Ukraine. Breeding birds were studied in Northern Jutland, Denmark and Chernobyl (51°16'N, 30°13'W) Ukraine, while non-breeding birds were studied during migration in Northern Jutland, Denmark and at Ottenby Bird Observatory (55°11'N, 23°56'W).

House sparrows were captured from a population placed at the university campus of Badajoz (38°52'N, 6°58'W), southwest Spain. Experimental studies (chapter II and chapter VI) were carried out in the aviaries of the Experimental Garden in the University of Extremadura.

Neotropical species studied in chapter IV were captured from different areas of Peru: Pantanos de Villa wetland Reserve, a RAMSAR protected area in the south of Lima including a complex of lagoons, pools and marsh areas of Pacific coast (12°12'S, 76°59'W; 10 masl), and Huánuco region, located between the eastern slope of the Andes Mountain Range and the Amazon plain (9°55'S, 76°14'W; 1894 masl).

Samples from house martins were obtained from a colony of in the surroundings of Badajoz (38°52′N, 7°05′W), south-western Spain (chapter V).

Behavioural variables (chapter I and II)

Six aspects of escape behaviour were assessed before the bird was ringed and released. Several of

these variables have been related to susceptibility to predation by cats and hawks (Møller et al. 2011). These six behavioural variables were defined as follows:

(1) Wriggle score. This variable scored how much the bird struggled while being held in a hand (a score of 0—no movement, 1—moves rarely, 2—moves regularly, but not always, 3—moves continuously). Individuals that wriggle more may more readily escape from a predator compared to individuals that stay calm.

(2) Biting. Whether the bird did not bite, it was given a score of 0, and if it did a score of 1. It is presume that a higher frequency of biting entails an elevated probability of escape from a predator because the predator loses its grip when re-directing its attention towards the biting prey.

(3) Feather loss. While the bird was handled, if it lost feathers it was given a score of 1, or 0 if it did not. Feather loss may result in predators losing their grip of a prey (Møller et al. 2006).

(4) Distress call. This variable is also called fear scream. While the bird was handled, if it gave a fear scream (a score of 1) or not (a score of 0). Birds giving fear screams attract the attention of secondary predators thereby increasing the probability of escape once captured (Högstedt 1983; Møller and Nielsen 2010).

(5) Tonic immobility. At the end of the above procedure the bird was placed, just before it was released, in the right hand on its back on the flat left hand. When the bird was lying still, the right hand was removed and the time until the bird righted itself and flew away was recorded. Tonic immobility is a standard measure of fear in poultry research with both environmental and genetic components (Hoagland 1928; Jones 1986; Boissy 1995; Forkman et al. 2007). More recently, Edelaar et al. (2012) showed that tonic immobility is related to personality and anti-predation behaviour because it is a measure of boldness toward predators. The longer time a bird stays, the higher its level of fear. Tonic immobility has a strongly bimodal distribution, with most individuals having tonic immobility of 0–5 s, but some 10–20% having 25–30 s as shown by (Møller et al. 2011).

(6) Alarm call. This variable measures whether the bird gave an alarm call (a score of 1) or not (a score of 0) when it departed. It has been suggested that the function of this call is to distract the predator or to warn conspecifics (Charnov and Krebs 1975; Platzen and Magrath 2004).

Molecular detection and sequencing of blood parasite infections (chapter I – VI)

In order to analyse the prevalence and genetic diversity of avian haemosporidian parasites, one microcapillary of blood (70 µl) was obtained from the brachial vein of each individual and stored in 500 µl of SET buffer (0.15 M NaCl, 0.05 Tris, 0.001 M EDTA, pH 8.0) until DNA extraction.

Haemosporidian parasites (*Plasmodium* spp. and *Haemoproteus* spp.) were detected from blood samples using molecular methods (Bensch et al. 2000; Waldenström et al. 2004). DNA from the avian blood samples were extracted in the lab using the standard phenol/chloroform/isoamylalcohol method (Sambrook et al. 2002). Diluted genomic DNA (25 ng/µl) was used as a template in a polymerase chain reaction (PCR) assay for detection of the parasites using nested PCR-protocols described by Waldenström et al. (2004). The amplification was evaluated by running 2.5 µl of the final PCR on a 2% agarose gel. All PCR experiments contained one negative control for every eight samples. In the very few cases of negative controls showing signs of amplification (never more than faint bands in agarose gels), the whole PCR-batch was run again to make sure that all positives were true. All positive amplifications were precipitated and sequenced in order to identify the species and lineage in each infection. The obtained sequences were edited, aligned and compared in a sequenced matrix using the program Bioedit (Hall 1999). Once *P. relictum* (SGS1 and GRW4 lineages) species were detected, a set of primers developed by Hellgren et al. (2013) were used in order to find out the MSP1 gene (chapter V). Similarly, samples from individuals infected with *P. relictum* (SGS1 and GRW4 lineages) were used in order to identify the chitinase gen (chapter IV).

Measurement of the feather grow rate (chapter VI)

With the aim to assess the feather growth rate, the right outermost tail feather was plucked from individuals. Feathers were stored in dry paper envelopes until laboratory analyses. The number of growth bars and the length of the right outermost rectrix feather were measured in a gel documentation system in the laboratory following the instructions from Shawkey et al. (2003). Once contrast and resolution were optimized, a digital image of the feather was obtained. The number of growth bars and the length of rectrix minus the calamus were measured using ImageJ software (Abràmoff et al. 2004). Feather grow rate for each individual was estimated by dividing the number of growth bars and the length of the right outermost rectrix feather.

Experimental treatments to reduce malaria infection (chapter II)

With the aim to reduce malaria infection, individuals were medicated with a combined treatment. Each bird of treatment group were sub-cutaneously injected with 0.02 mg of Primaquine + 1.4 mg of Chloroquine diluted in 0.2 ml of saline solution (Remple 2004) and control individuals (N = 40) were injected with 0.2 ml phosphate buffered saline (PBS). A Malarone™ treatment were also provide with fixed – dose combination of 250 mg of atovaquone and 100 mg of proguanil hydrochloride to individuals of the treatment group (Palinauskas et al. 2009). The same quantity of water was provided in the dispensers of the control group, but without Malarone. The experiment was run over 2 months.

Experimental inoculation of malaria infection (chapter VI)

Natural infected birds were kept as donors for the experiment. Infected group (N = 10), was experimentally infected by intramuscularly inoculation of 250 uL of malaria infected blood mixture (100 uL blood from infected house sparrow donor, 25 uL 3.7 % sodium citrate, 125 uL 0.9% buffered saline) in the pectoral muscle (Palinauskas et al. 2008). Control group (N = 8), was inoculated with 250 uL of PBS.

MAIN RESULTS AND CONCLUSIONS

1. *Host escape behaviour and blood parasite infections in birds*

Intense escape behavior was positively related to prevalence of infection with *Haemoproteus* and *Leucocytozoon*, whereas that was not the case for *Plasmodium*. Species emitting more frequently fear screams and struggling more when held in a hand showed higher prevalence of *Haemoproteus* and *Leucocytozoon* (Fig. 4). These results suggest that species with a higher intensity of escape behaviour have higher prevalence of blood parasites showing that there is a correlation between escape behaviour and blood parasite infection.

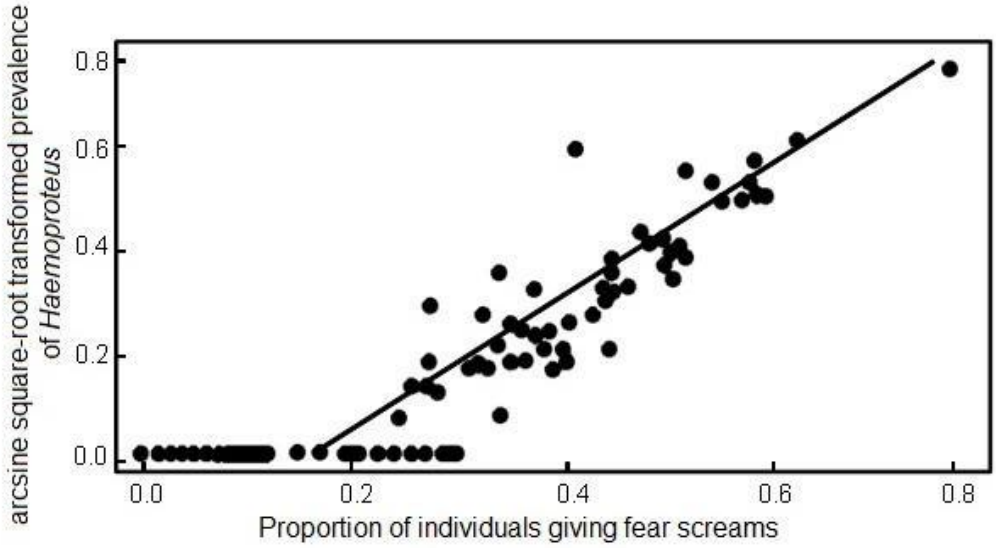
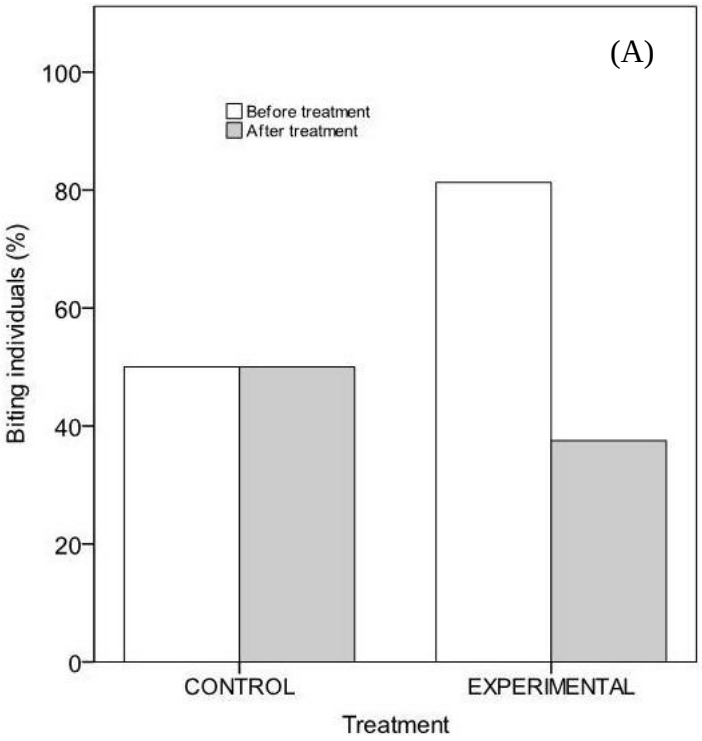


Figure 4. Proportion of individuals giving a fear scream in different species of birds in relation to prevalence of *Haemoproteus*. The line is the linear regression line.

2. *Do malaria parasites manipulate the escape behaviour of their avian hosts? An experimental study*

The prevalence of malaria infection was reduced in infected individuals treated with primaquine and chloroquine. Once the infection was cleared, the biting rate of these medicated sparrows significantly decreased. Additionally, these birds also spent more time in tonic immobility before flying away (Fig. 5). These outcomes imply that the experimental reduction of malaria parasites provoked a decrease in the intensity of escape behaviour, suggesting that malaria parasites may manipulate the escape behaviour of their avian hosts. Therefore, malaria parasite could increase the likelihood of individuals escaping from predators, but also would benefit the parasite by increasing its transmission opportunities.



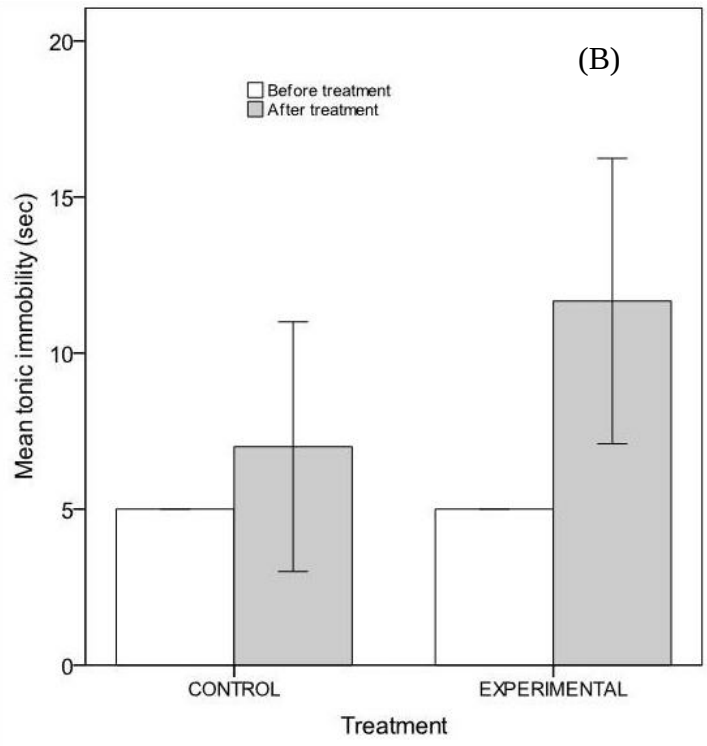


Figure 5. Escape behaviour before and after the treatment in infected house sparrows. (A) Biting individual house sarrows (%) in control and experimental groups before and after the anti-malaria treatment. (B) Mean tonic immobility (seconds) in control and experimental groups of house sparrows before and after anti-malaria treatment.

3. Invasive avian malaria as an emerging parasitic disease in native birds of Peru

The overall prevalence of avian malaria and related haemosporidian found in Neotropical birds was 32.4%. 12 out of 18 native bird species were infected with haemosporidian parasites. The pathogen *Plasmodium relictum* SGS1 was widespread and the most prevalent parasite found (39 % of the total infections), infecting eight host species in both localities (Table 1). As far as we know, this is the first report of this invasive pathogen in the mainland Americas, thus representing a possible menace to over one-third of all bird species in the world.

Lineage	Genus	GenBank#	Prevalence of total infection (%)	Localities	N alternative host
BAEBIC02	P	AF465555	15.15	HUAN	1
CHLOP01	H	JQ764618	18.18	HUAN	1
PHPAT01	P	EF153642	3.03	HUAN	1
PYERY01	H	AY172842	3.03	HUAN	1
SERCIN01	H	KF482344	3.03	HUAN	1
SGS1	P	AF495571	39.40	HUAN, PV	8
STTA17H	H	JN819389	9.09	HUAN, PV	2
TACHURIS01	P	KF482356	3.03	PV	1
TROGLODY01	P	KF482358	3.03	PV	1
ZOCAP01	H	KF482358	3.03	HUAN	1

Table 1. Lineage names, parasite genus (H *Haemoproteus*, P *Plasmodium*), GenBank accession numbers, prevalence of total infection, localities where have been sampled (Huan *Huanuco*, PV *Pantanos de Villa*) and number of alternative host where each blood parasite cyt b lineage was found.

4. Molecular identification of the chitinase genes in Plasmodium relictum

Chitinase gene was identified in two mitochondrial lineages of *Plasmodium relictum* (SGS1 and GRW4). These mitochondrial lineages showed both the long (*Pr*CHT1) and the short (*Pr*CHT2) copy of the chitinase gene (Fig. 6). The genetic differences found in the long copy of the chitinase gene between SGS1 and GRW4 were higher than the difference observed for the cytochrome b gene. Because of this high variability, the chitinase gene can be used for epidemiological studies of malaria parasite, similarly to previous studies (Hellgren et al. 2014). This gene can provide new information about the distribution and dynamics infection of these two cytochrome b lineages among populations. Moreover, the identification of both copies in *P. relictum* sheds light on the phylogenetic relationship of the chitinase gene in the genus *Plasmodium*, supporting the hypothesis that avian malaria parasites are the antecessor of mammal malaria parasites.

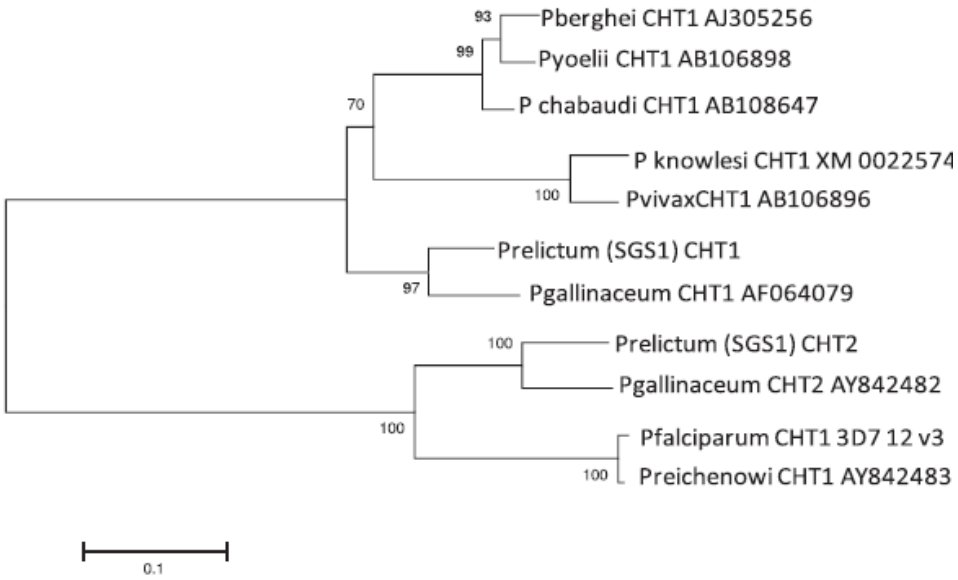


Figure 6. Maximum likelihood consensus phylogeny using midpoint rooting of the translated CHT1 (long copy) gene and CHT2 (short copy) from 10 different malaria species. Numbers in branches represent bootstrap values based on 200 iterations.

5. Detection of the merozoite surface protein 1 (msp1) gene in house martins

Three juvenile house martins were infected by *Plasmodium relictum* SGS1. All these juveniles were infected with MSP1 allele Pr2 (Fig. 7), thus showing that this allele in actively transmitted in Europe. This is the first report showing an active transmission of avian malaria parasites in house martins in Europe. Moreover, most of the adult house martins infected with SGS1 also showed the same MSP1 allele than juveniles (Pr2). Additionally, two adult house martins were infected with MSP1 alleles Pr1 (SGS1) and Pr4 (GRW4), but these alleles were not found infecting juvenile house martins (Fig. 7). These findings suggest that most of the house martin population may get the infection by SGS1 on Europe, although we cannot discard that SGS1 Pr2 could also be transmitted in Africa. All these results show that house martins are exposed to two different parasite fauna of *P. relictum*.

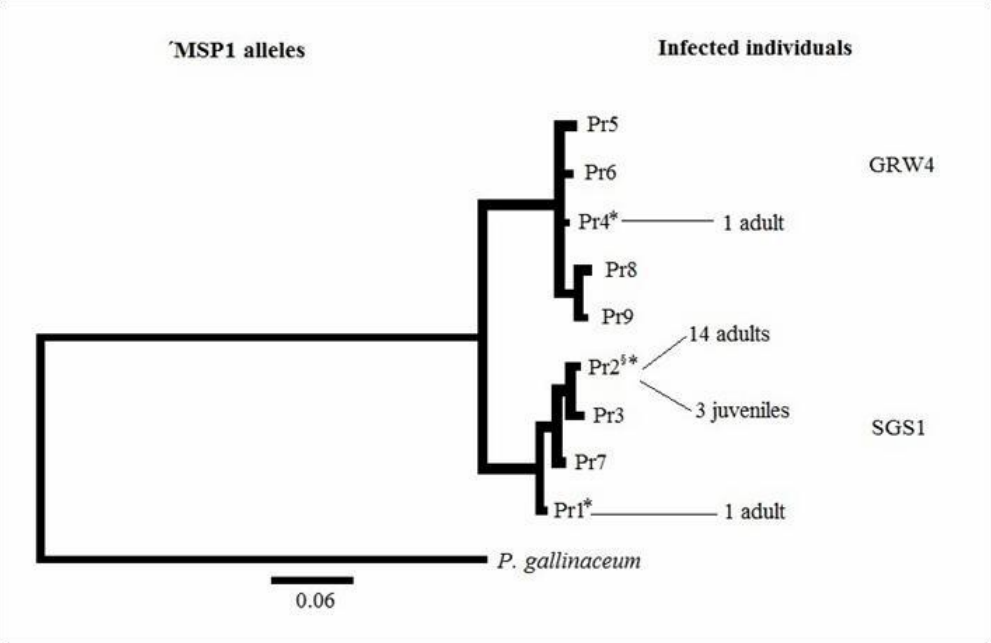
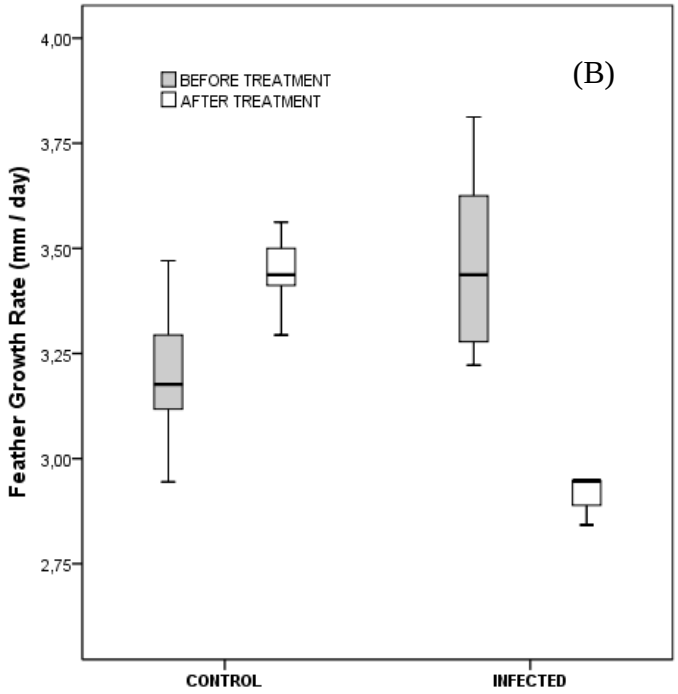


Figure 7. Phylogenetic relationship between all the MSP1_b14 alleles detected to date (Hellgren et al. 2015) and number of individuals (adults or juveniles) infected by these alleles. * and § represent confirmed active transmission in Africa and Europe, respectively (Hellgren et al. 2015).

6. *Malaria infection may affect negatively feather growth rate in the ubiquitous sparrow*

Malaria parasite (*Plasmodium relictum*) was identified as a factor provoking a decrease in feather grow rate in both natural-infected and experimental-infected individuals (Fig. 8). These outcomes demonstrate the negative effects of malaria parasites on the feather growth rate of house sparrows under natural and experimental conditions.



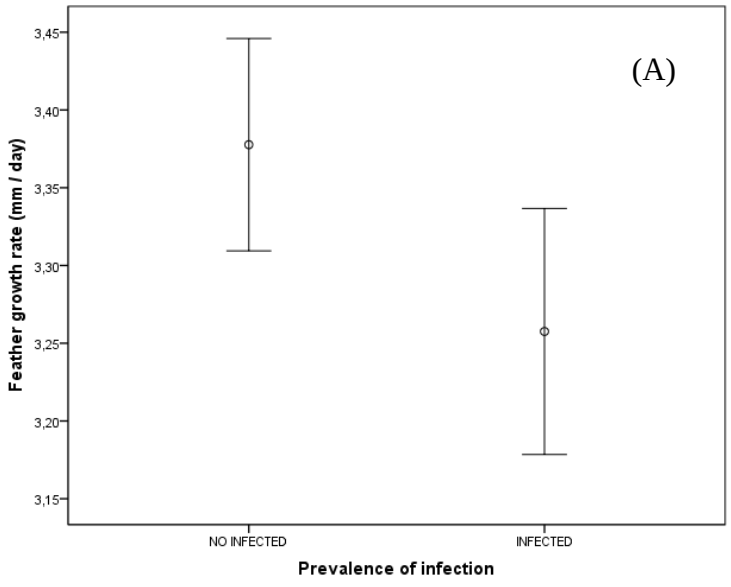


Figure 8. Differences in feather grow rate between natural-infected and non infected house sparrows (A) and between experimental-infected and control house sparrows (B).

REFERENCES

Abràmoff MD, Magalhães PJ, Ram SJ (2004) Image Processing with ImageJ. Biophotonics Int 11:36–41.

Allander K, Sundberg J (1997) Temporal Variation and Reliability of Blood Parasite Levels in Captive Yellowhammer Males *Emberiza citrinella*. J Avian Biol 28:325–330.

Álvarez N, Borrero R, Reyes F, et al. (2009) Mechanisms of evasion and persistence of *Mycobacterium tuberculosis* during the latencystate and potential strategies to control latent infection. Vaccimonitor 18:74–82.

Asghar M, Hasselquist D, Bensch S (2011) Are chronic avian haemosporidian infections costly in wild birds? J Avian Biol 42:530–537.

Beadell JS, Ishtiaq F, Covas R, et al. (2006) Global phylogeographic limits of Hawaii's avian malaria. Proc Biol Sci 273:2935–2944.

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

Bensch S, Stjernman M, Hasselquist D, et al. (2000) Host specificity in avian blood parasites: a study of *Plasmodium* and *Haemoproteus* mitochondrial DNA amplified from birds. Proc Biol Sci 267:1583–1589.

Blumstein DT, Patton ML, Saltzman W (2006) Faecal glucocorticoid metabolites and alarm calling in free-living yellow-bellied marmots. Biol Lett 2:29–32.

Boissy A (1995) Fear and fearfulness in animals. Q Rev Biol 70:165–191.

Bush A, Fernandez J, Esch G, Seed J (2001) Parasitism. The diversity and ecology of animal parasites. Zoolog Sci. New York , Cambridge.

Butler M, Johnson AS (2004) Are melanized feather barbs stronger? J Exp Biol 207:285–293.

Charnov LE, Krebs RJ (1975) The evolution of alarm calls: altruism or manipulation? Am Nat 109:107–112.

Clayton D, Moore J (1997) Host-parasite evolution: general principles and avian model. Oxford Univ. Press, England

Cohen J (2003) Patterns Human population: the next half century. Science 302:1172–1175.

Coon CAC, Martin LB (2014) Patterns of haemosporidian prevalence along a range expansion in introduced Kenyan house sparrows *Passer domesticus*. J Avian Biol 45:34–42.

Cornet S, Nicot A, Rivero A, Gandon S (2013) Malaria infection increases bird attractiveness to uninfected mosquitoes. Ecol Lett 16:323–329.

Cramp S, Perrins CM (1994) Handbook of the birds of Europe, the Middle East, and North Africa: the birds of the Western Palearctic volume VIII: crows to finches. hand birds Eur Middle East North Africa 5:41.

DesRochers DW, Reed JM, Awerman J, et al. (2009) Exogenous and endogenous corticosterone alter feather quality. Comp Biochem Physiol - A Mol Integr Physiol 152:46–52.

Dunn JC, Cole EF, Quinn JL (2011) Personality and parasites: sex-dependent associations between avian malaria infection and multiple behavioural traits. Behav Ecol Sociobiol 65:1459–1471.

Edelaar P, Serrano D, Carrete M, et al. (2012) Tonic immobility is a measure of boldness toward predators: an application of Bayesian structural equation modeling. Behav Ecol 23:619–626.

Escalante AA, Ayala FJ (1994) Phylogeny of the malarial genus *Plasmodium*, derived from rRNA gene sequences. Proc Natl Acad Sci USA 91:11373–11377.

Forkman B, Boissy A, Meunier-Salaün MC, et al. (2007) A critical review of fear tests used on cattle, pigs, sheep, poultry and horses. Physiol Behav 92:340–374.

Garamszegi LZ (2011) Climate change increases the risk of malaria in birds. Glob Chang Biol 17:1751–1759.

Garcia-Longoria L, Garamszegi LZ, Møller AP (2014) Host escape behaviour and blood parasite infections in birds. Behav Ecol 25:890-900.

Gardner MJ, Hall N, Fung E, et al. (2002) Genome sequence of the human malaria parasite *Plasmodium falciparum*. Nature 419:498–511.

George A (2009) 7 billion and counting. New Scientist 203:34–35.

Gerold P, Schofieldb L, Blackman MJ, et al. (1996) Structural analysis of the glycosyl-phosphatidylinositol membrane anchor of the merozoite surface proteins-1 and -2 of *Plasmodium falciparum*. Mol Biochem Parasitol 75:131–143.

Ghosh A, Edwards MJ, Jacobs-Lorena M (2000) The journey of the malaria parasite in the mosquito: hopes for the new century. Parasitol Today 16:196–201.

Ginn HB, Melville D (1983) Moult in birds (BTO Guide 19). J Ornithol 116:263–280.

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

Hamilton WD, Zuk M (1982) Heritable true fitness and bright birds: a role for parasites? Science 218:384–387.

Hatcher MJ, Dunn AM (2011) Parasites in Ecological Communities. From Interactions to Ecosystems. Cambridge, UK. Cambridge University Press.

Hediger H (1934) Zur Biologie and Psychologie der Flucht bei Tieren. Biol Zent Bl 54:21–40.

Hellgren O, Kutzer M, Bensch S, et al. (2013) Identification and characterization of the merozoite surface protein 1 (msp1) gene in a host-generalist avian malaria parasite, *Plasmodium relictum* (lineages SGS1 and GRW4) with the use of blood transcriptome. Malar J 12:381.

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

Hellgren O, Carter A, Bensch S, et al. (2015) Global phylogeography of the avian malaria pathogen *Plasmodium relictum* based on MSP1 allelic diversity. Ecography doi: 10.1111/ecog.01158.

Hoagland H (1928) On the mechanism of tonic immobility in vertebrates. J Gen Physiol 11:715–741.

Högstedt G (1983) Adaptation Unto Death: Function of Fear Screams. Am Nat 121:562–574.

Howe L, Castro IC, Schoener ER, et al. (2012) Malaria parasites (*Plasmodium* spp.) infecting introduced, native and endemic New Zealand birds. Parasitol Res 110:913–923.

Jones RB (1986) The tonic immobility reaction of the domestic fowl: a review. World Poult Sci J 42:82–96.

Jovani R, Blanco G (2000) Resemblance within flocks and individual differences in feather mite abundance on long-tailed tits. Ecoscience 7:428–432.

Kadota K, Ishino T, Matsuyama T, et al. (2004) Essential role of membrane-attack protein in malarial transmission to mosquito host. Proc Natl Acad Sci USA 101:16310–16315.

Kang JM, Ju HL, Kang YM, et al. (2012) Genetic polymorphism and natural selection in the C-terminal 42 kDa region of merozoite surface protein-1 among *Plasmodium vivax* Korean isolates. Malar J 11:206.

Klaassen M (1995) Moult and basal metabolic costs in males of two subspecies of stonechats: the European *Saxicola torquata rubicula* and the East African *S. t. axillaris*. Oecologia 104:424–432.

Koella JC, Rieu L, Paul REL (2002) Stage-specific manipulation of a mosquito's host-seeking behavior by the malaria parasite *Plasmodium gallinaceum*. Behav Ecol 13:816–820.

Lees E (1962) The incidence of parasites in a particular frog population. Parasitology 52:95–102.

Lefèvre T, Roche B, Poulin R, et al. (2008) Exploiting host compensatory responses: the "must" of manipulation? Trends Parasitol 24:435–439.

Li F, Patra KP, Vinetz JM (2005) An anti-chitinase malaria transmission-blocking single-chain antibody as an effector molecule for creating a *Plasmodium faltiparum*-refracory mosquito. J Infect Dis 192:878–887.

Li F, Templeton TJ, Popov V, et al. (2004) *Plasmodium* ookinete-secreted proteins secreted through a common micronemal pathway are targets of blocking malaria transmission. J Biol Chem 279:26635–26644.

Lima MR, Simpson L, Fecchio A, Kyaw CM (2010) Low prevalence of haemosporidian parasites in the introduced house sparrow (*Passer domesticus*) in Brazil. Acta Parasitol 55:297–303.

Lind J (2001) Escape flight in moulting Tree Sparrows (*Passer montanus*). Funct Ecol 15:29–35.

Loiseau C, Zoorob R, Robert A, et al. (2011) *Plasmodium relictum* infection and MHC diversity in the house sparrow (*Passer domesticus*). Proc Biol Sci 278:1264–1272.

Lowe S, Browne M, Boudjelas S, De Poorter M (2000) 100 of the World's Worst Invasive alien species a selection from the global invasive species database. IUCN/SSC Invasive Species Specialist Group (ISSG) 12:12.

Loye JE, Zuk M (1991) Bird-parasite interactions: ecology, evolution and behaviour, Oxford Uni.

Marzal A, Asghar M, Rodríguez L, et al. (2013a) Co-infections by malaria parasites decrease feather growth but not feather quality in house martin. J Avian Biol 44:437–444.

Marzal A, Bensch S, Reviriego M, et al. (2008) Effects of malaria double infection in birds: one plus one is not two. J Evol Biol 21:979–987.

Marzal A, Reviriego M, Hermosell IG, et al. (2013b) Malaria infection and feather growth rate predict reproductive success in house martins. Oecologia 171:853–861.

Marzal A, Ricklefs RE, Valkiūnas G, et al. (2011) Diversity, loss, and gain of malaria parasites in a globally invasive bird. PLoS One 6:8.

Merilä J, Andersson M (1999) Reproductive effort and success are related to haematozoan infections in blue tits. Ecoscience 6:421–428.

Merino S, Moreno J, Jose J, Arriero E (2000) Are avian blood parasites pathogenic in the wild? A medication experiment in blue tits (*Parus caeruleus*). R Soc 9:2507–2510.

Møller AP, Erritzøe J (1998) Host immune defence and migration in birds. Evol Ecol 12:945–953.

Møller AP, Christiansen SS, Mousseau TA (2011) Sexual signals, risk of predation and escape behavior. Behav Ecol 22:800–807.

Møller AP, Nielsen JT (2010) Fear screams and adaptation to avoid imminent death: effects of genetic variation and predation. Ethol Ecol Evol 22:183–202.

Møller AP, Nielsen JT, Erritzøe J (2006) Losing the last feather: feather loss as an antipredator adaptation in birds. Behav Ecol 17:1046–1056.

Moore J (2002) Parasites and the Behavior of Animals. J Parasitol 88:1214.

Morales J, Moreno J, Merino S, et al. (2007) Early moult improves local survival and reduces reproductive output in female pied flycatchers. Ecoscience 14:31–39.

Moreno-Rueda G (2010) Uropygial gland size correlates with feather holes, body condition and wingbar size in the house sparrow *Passer domesticus*. J Avian Biol 41:229–236.

Murphy ME (1996) Energetics and nutrition of molt. Avian Energ New York: Plenum Press.

Murphy ME, Taruscio TG (1995) Sparrows increase their rates of tissue and whole-body protein synthesis during the annual molt. Comp Biochem Physiol - A Physiol 111:385–396.

Nava MP, Veiga JP, Puerta M (2001) White blood cell counts in house sparrows (*Passer domesticus*) before and after moult and after testosterone treatment. Can J Zool-Rev Can Zool 79:145–148.

Noranate N, Prugnolle F, Jouin H, et al. (2009) Population diversity and antibody selective pressure to *Plasmodium falciparum* MSP1 block2 locus in an African malaria-endemic setting. BMC Microbiol 9:219.

Pacheco MA, Reid MJC, Schillaci MA, et al. (2012) The origin of malarial parasites in orangutans. PLoS One 7:3.

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

Palinauskas V, Valkiūnas G, Bolshakov C V, Bensch S (2008) *Plasmodium relictum* (lineage P-SGS1): effects on experimentally infected passerine birds. Exp Parasitol 120:372–380.

Palinauskas V, Valkiūnas G, Krizanauskiene A, et al. (2009) *Plasmodium relictum* (lineage P-SGS1): Further observation of effects on experimentally infected passeriform birds, with remarks on treatment with Malarone. Exp Parasitol 123:134–139.

Platzen D, Magrath RD (2004) Parental alarm calls suppress nestling vocalization. Proc Biol Sci 271:1271–1276.

Podmokła E, Dubiec A, Drobniak SM, et al. (2014) Avian malaria is associated with increased reproductive investment in the blue tit. J Avian Biol 45: 219-224.

Poulin R (2010) Parasite manipulation of host behavior: an update and frequently asked questions. Adv Study Behav 41:151–186.

Proctor N, Lynch P (1994) Manual of Ornithology: Avian Structure and Function. Am Sci 82:288–289.

Remple DJ (2004) Intracellular Hematozoa of Raptors: A Review and Update. J Avian Med Surg 18:75–88.

Sambrook J, Fritsch EF, Maniatis T (2002) Molecular cloning: a laboratory manual, cold sprin. Cold Spring Harb Lab 3:2344.

Santiago-Alarcon D, Havelka P, Pineda E, et al. (2013) Urban forests as hubs for novel zoonosis: blood meal analysis, seasonal variation in Culicoides (Diptera: *Ceratopogonidae*) vectors, and avian haemosporidians. Parasitology 24:1–12.

Schmid-Hempel P (2011) Evolutionary parasitology: The integrated study of infections, immunology, ecology and genetics. Trends Parasitol 27:233–237.

Shahabuddin M, Toyoshima T, Aikawa M, Kaslow DC (1993) Transmission-blocking activity of a chitinase inhibitor and activation of malarial parasite chitinase by mosquito protease. Proc Natl Acad Sci USA 90:4266–4270.

Shawkey MD, Beck ML, Hill GE (2003) Use of a gel documentation system to measure feather growth bars. J F Ornithol 74:125–128.

Sieber KP, Huber M, Kaslow D, et al. (1991) The peritrophic membrane as a barrier: its

penetration by *Plasmodium gallinaceum* and the effect of a monoclonal antibody to ookinetes. Exp Parasitol 72:145–156.

Snow RW, Guerra CA, Noor AM, et al. (2005) The global distribution of clinical episodes of *Plasmodium falciparum* malaria. Nature 434:214–217.

Summers-Smith J (1988) The Sparrows: A Study of the Genus Passer. T. & A. D. Poyser Ltd, Calton.

Tanabe K, Mita T, Palacpac NMQ, et al. (2013) Within-population genetic diversity of *Plasmodium falciparum* vaccine candidate antigens reveals geographic distance from a Central sub-Saharan African origin. Vaccine 31:1334–1339.

Tarello W (2007) Clinical signs and response to primaquine in falcons with *Haemoproteus tinnunculi* infection. Vet Rec 161:204–205.

Tsai YL, Hayward RE, Langer RC, et al. (2001) Disruption of *Plasmodium falciparum* chitinase markedly impairs parasite invasion of mosquito midgut. Infect Immun 69:4048–4054.

Vágási CI, Pap PL, Vincze O, et al. (2012) Haste makes waste but condition matters: Molt rate-feather quality trade-off in a sedentary songbird. PLoS One 7:7.

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

Valkiūnas G (2005) Avian malaria parasites and other Haemosporidia, Boca Raton.

Valkiūnas G, Tadas Z, Shapoval AP, Iezhova TA (2006) Effect of *Haemoproteus belopolskyi* (Haemosporida: *Haemoproteidae*) on body mass of the blackcap *Sylvia atricapilla*. Am Soc Parasitol 92:1123–1125.

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

Williams EV, Swaddle JP (2003) Moult, flight performance and wing beat kinematics during take-off in European starlings *Sturnus vulgaris*. J Avian Biol 34:371–378.

Host escape behaviour and blood parasite infections in birds

I

Feet, why do I want if I have wings to fly
Frida Kahlo

Un comportamiento activo o arriesgado puede conllevar un mayor contacto con depredadores así como encuentros más frecuentes con vectores de parásitos sanguíneos mediante la exploración de diferentes hábitats. De esta manera, creemos que el comportamiento anti-depredador, conocido como comportamiento de escape, llevado a cabo por el ave cuando ha sido capturada por un humano estaría correlacionado con el riego de infecciones parásitas, donde especies más audaces mostrarían más parásitos que especies menos propensas al riesgo. En este estudio comprobamos si especies con una mayor intensidad de comportamiento de escape tendrían mayor prevalencia de parásitos sanguíneos, en concreto haemosporidios. Encontramos que el comportamiento de escape estuvo relacionado de manera intermedia y positiva con la prevalencia de infección de *Haemoproteus* y *Leucocytozoon*, sin embargo en el caso del género más virulento *Plasmodium* no encontramos ninguna relación. Especies que visitaban mayor número de hábitats mostraron una mayor prevalencia de parásitos sanguíneos que especies que se visitaban un menor número de hábitats. Además, algunas variables del comportamiento de escape estuvieron correlacionadas de manera intermedia con la exploración de hábitats y con el hecho de ser coloniales. No encontramos ningún patrón de correlación entre la mayoría de las variables comportamentales y la distancia de iniciación al vuelo, otro comportamiento anti-depredador muy común. De esta manera, el comportamiento que se muestra cuando el depredador se está acercando o cuando el ave está capturada por un humano representan diferentes ejes de comportamiento anti-depredador. Estos resultados concuerdan con la hipótesis de que el comportamiento de escape está relacionado con el riesgo de infección de parásitos sanguíneos mediado parcialmente por el efecto que puede causar el visitar un mayor número de hábitats.

Palabras clave: parásitos sanguíneos, colonial, comportamiento de escape, grito de alarma, innovación en la alimentación, hábitat, interacción hospedador-parásito, depredación.

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Original Article

Host escape behavior and blood parasite infections in birds

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Introduction

Behavioral traits often vary considerably both between and within individuals (Bell et al. 2009), determining whether an individual ends up as a prey or survivor. Antipredator behavior is highly consistent in the presence of a predator across time and contexts (Dammhahn and Almeling 2012). Numerous studies have demonstrated that predators impose important selection pressures on prey (Endler 1986; Clinchy et al. 2004; Roulin and Wink 2004). The first means of avoidance of predation is fleeing when a predator is approaching, as reflected by flight initiation distance in birds (Hediger 1934; Blumstein 2006). The second means of avoidance of predation includes a suite of escape behaviors that are used once captured by a predator (Møller et al. 2011). Such escape behaviors include the intensity with which a captured individual wriggles to escape, whether an individual bites or not, whether it loses feathers (Møller et al. 2006), limbs, or a tail and thereby manages to escape, whether it emits alarm or distress calls (Högstedt 1983; Møller and Nielsen 2010), and the duration of tonic immobility that can be considered a behavior akin to feigning death. For example, prey individuals may elicit loud and piercing fear screams that may be directed either to other predators or to conspecifics thereby facilitating escape (Högstedt 1983). Møller et al. (2011) demonstrated that individuals particularly susceptible to predation showed higher tonic immobility and wriggled more when a human captured them. Such escape behavior has been related to susceptibility to predation, implying that the use of antipredator behavior could increase the probability of escape from a predator once captured (Møller et al. 2011). The different components of escape behavior albeit species specific are mostly independent of each other, and they are generally not related to flight initiation distance (Hediger 1934; Blumstein 2006).

Parasites constitute another major cause of mortality in addition to predation. It has been demonstrated that the presence of predators may interact indirectly with parasites, causing stress to birds and reducing immune function of the host. Navarro et al.

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(2004), for example, showed that predators caused a reduction in T-cell-mediated immune response in house sparrows, *Passer domesticus*, probably because individuals exposed to predators suffered stress-mediated reductions in immune function. Therefore, individuals or species exposed to predation had a reduced ability to cope with infection as revealed by an increase in the prevalence and the intensity of blood parasites. Moreover, Møller and Nielsen (2007) showed that species with high parasite loads were worse at escaping from predators. This reveals a potential underlying mechanism that links predation to prevalence of blood parasites. We studied hemosporidian parasites (*Plasmodium*, *Leucocytozoon*, and *Haemoproteus*) that are widespread and harmful (Valkiūnas 2005) infecting squamate reptiles, turtles, birds, and mammals and using at least 7 families of dipteran vectors (Levine 1988; Martinsen et al. 2008). Hemosporidian parasites have important effects on the life history of hosts by reducing survival (Dawson and Bortolotti 2000; Breman 2001; Valkiūnas 2005), body condition (Valkiūnas et al. 2006; Palinauskas et al. 2008), and reproductive success (Merino et al. 2000; MacDougall-Shackleton et al. 2002; Marzal et al. 2005; Tomás et al. 2007). Hence, a considerable number of studies have focused on the effects of these protozoans in birds (Hellgren et al. 2009; Dimitrov et al. 2010; Garamszegi 2011; Lachish et al. 2011; Mostowy and Engelstädter 2011; Cornet et al. 2013). These blood parasites show a complex life cycle, where the presence of a vector transmitting the infection is required (Valkiūnas 2005). The wide distribution of these blood parasites also implies the presence of vectors (biting midges, louse flies, black flies, and a large number of mosquito species) transmitting the disease (Levine 1988; Valkiūnas 2005; Martinsen et al. 2008). The vector life cycle is very sensitive to changes in weather conditions (Patz and Olson 2006). The abundance and diversity of these vectors vary among habitats depending mainly on weather (Wood et al. 2007; Paaijmans et al. 2009; Garamszegi 2011). Moreover, ecological conditions can play an important role in affecting the prevalence of blood parasites if migration causes stress and hence stress-induced immunosuppression (Waldenström et al. 2002). Alternatively, migration results in hosts encountering a greater diversity of habitats with a greater diversity of parasites, as we have argued for habitat selection here. Finally, there is a positive correlation between group size and prevalence of blood parasites (Rifkin et al. 2012). Hence, habitat choice, migratory behavior, and coloniality could affect prevalence of blood parasites.

Species that move between different habitats can be considered habitat generalists, whereas species restricted to one or a few habitats are considered habitat specialists, with the former taking higher risks when visiting novel habitats than the latter remaining in the same habitat (Belliure et al. 2000). If each habitat has a nonzero probability of harboring a parasite, individuals that are frequenting several habitats will experience an elevated probability of acquiring more parasites than individuals that remain in a single habitat. Specialists may also be able to defend themselves against parasites once infected, while that may be the case less frequently for generalists because the defense against 1 type of parasite may not be efficient against another. Thus, host species that are habitat generalists may more readily encounter a vector infected by blood parasites than habitat specialists, and bolder individuals may differ in risky behavior from more fearful individuals. Bold individuals that more actively explore their environment may more readily come into contact with infectious stages of parasites or vectors that transmit parasites (Wilson et al. 1993; Moore 2002; Barber and Dingemanse 2010; Boyer et al. 2010). Thus, behavior of hosts may play an important role in determining whether an individual becomes infected or not. Furthermore, species with higher loads of blood parasites more frequently exploit different kinds of resources than less infected species and hence have a larger feeding innovation rate (Garamszegi et al. 2007). If escape behavior is the underlying mechanism that causes certain individuals to visit a larger number of habitats and become exposed more frequently to parasites, we might expect an association between this antipredator behavior and risk of parasitism.

The objective of this study was to test whether species with high prevalence of blood parasites differed in escape behavior from species with low prevalence. This objective rests on 2 nonexclusive hypotheses: 1) Predators impose parallel selection on both parasite load (affecting immune defenses indirectly) and escape behavior, causing a correlation between escape behavior and parasitic load, and 2) habitat generalists with pronounced escape behavior (e.g., higher frequency of biting or vigorous wriggle) are more exposed to vector-transmitted parasites than habitat specialists, thereby causing differences in prevalence of blood parasites. We also expect that escape behavior differs among species because species occupy different niches and are at risk from different types of predators because their hunting and prey handling strategies vary among niches. Firstly we tested if escape behavior was related to flight initiation distance, with the aim to determine whether they represent different components of antipredator behavior. Secondly we tested if prevalence of blood parasites in different host species is related to differences in escape behavior taking habitat generalism, habitat choice, migration, and coloniality into account. A positive correlation between prevalence of blood parasites and a high intensity of escape behavior would be consistent with the hypothesis that differences in ranging behavior cause differences in prevalence. Similarly, if there were a correlation between prevalence of blood parasites and habitat, we would expect that differences in vector abundance could cause differences in prevalence. Moreover, a correlation between migration or coloniality and prevalence of blood parasites would be expected when behavioral variables were controlled statistically. Sol et al. (2005) showed that the frequency of feeding innovations is positively related to invasiveness, suggesting a connection between feeding innovation rate and habitat exploration. Thirdly we tested whether intensity of escape behavior was correlated with habitat exploration through cognitive abilities as reflected by the relative frequency of feeding innovations. Previous studies have shown that colonial breeders may experience higher risks of predation (Marchant and Higgins 1993; Dall and Griffith 2014), and as a result such species should show higher intensity of escape behavior (Sims et al. 2008; Sorace and Gustin 2009;Ibáñez-Alamo and Soler 2010; Møller and Ibáñez-Alamo 2012). Lastly 4), we tested whether intensity of escape behavior was correlated with coloniality.

Materials and Methods

Behavioral variables

Information on escape behavior was derived from a previous study of extensive capture of birds during 2010–2012 in Denmark, Sweden, and Ukraine, see Møller et al. (2011) for study sites, timing, and extent of sampling and permits. It is common in comparative analyses to include data obtained in already published studies. Birds were exclusively captured for the study presented in Møller et al. (2011), and no additional captures were made for the present study. In this study (Møller et al. 2011), 6 aspects of escape behavior

were assessed before the bird was ringed and released. Several of these variables have been related to susceptibility to predation by cats and hawks (Møller et al. 2011). These 6 behavioral variables were defined as follows:

(1) Wriggle score. We scored how much the bird struggled while being held in a hand (a score of 0—no movement, 1—moves rarely, 2—moves regularly, but not always, 3—moves continuously). Individuals that wriggle more may more readily escape from a predator compared with individuals that stay calm.
(2) Biting. Whether the bird did not bite, when we held our right-hand index finger in front of the beak, we gave a score of 0, and if it did a score of 1. We presume that a higher frequency of biting entails an elevated probability of escape from a predator because the predator looses its grip when redirecting its attention toward the biting prey.
(3) Feather loss. While the bird was handled, if it lost feathers we gave a score of 1, or 0 if it did not. Feather loss may result in predators loosing their grip of a prey (Møller et al. 2006).
(4) Distress call. This variable is also called fear scream. While the bird was handled, if it gave a fear scream (a score of 1) or not (a score of 0). Subsequently, we use the term fear scream for this variable. Birds giving fear screams attract the attention of secondary predators thereby increasing the probability of escape once captured (Högstedt 1983; Møller and Nielsen 2010).
(5) Tonic immobility. At the end of the above procedure we placed the bird, just before it was released, with our right hand on its back on our flat left hand. When the bird was lying still, we removed the right hand and recorded the time until the bird righted itself and flew away. We allowed tonic immobility up to 30 s, and if the bird had not left yet, we terminated the trial. Tonic immobility is a standard measure of fear in poultry research with both environmental and genetic components (Hoagland 1928; Jones 1986; Boissy 1995; Forkman et al. 2007). More recently, Edelaar et al. (2012) showed that tonic immobility is related to personality and antipredation behavior because it is a measure of boldness toward predators. The longer time a bird stays, the higher its level of fear. Tonic immobility has a strongly bimodal distribution, with most individuals having tonic immobility of 0–5 s, but some 10–20% having 25–30 s as shown by Møller et al. (2011).
(6) Alarm call. When the bird departed from our hand whether it gave an alarm call (a score of 1) or not (a score of 0). It has been suggested that the function of this call is to distract the predator or to warn conspecifics (Charnov and Krebs 1975; Platzen and Magrath 2004).

We recorded body mass using a Pesola spring balance (accuracy 0.1 g) and included this variable in all models to account for the fact that larger individuals have a larger body surface and hence may encounter more vectors transmitting blood parasites (Valkiūnas 1987).

Flight initiation distance

We used a database on flight initiation distances in birds collected by A.P.M. during 2006–2012 (Møller 2008a). In brief, A.P.M. walked at a normal speed toward the individual bird, once a bird had been located with a pair of binoculars, while recording the number of steps between where A.P.M. was at the moment of birds' takeoff and the position of the bird. The number of steps approximately equals the distance in meters (Møller 2008b), and it is strongly correlated with the measured distance using a Nikon Forestry 550 hypsometer (Møller AP, unpublished data). The distance at which the individual took flight was recorded as the flight initiation distance, while the starting distance was defined as the distance from where A.P.M. started walking toward the bird to the position of the bird. The height above ground was recorded to the nearest meter. While recording these flight initiation distances, A.P.M. also recorded date, time of day, and sex if possible, A.P.M. recorded in total 2298 individuals belonging to 44 bird species. Further details and cross-validation of these data among seasons, years, countries, and observers have already been reported elsewhere (Møller 2008b).

Feeding innovation rate

With the aim to test whether habitat exploration through cognitive abilities is related to escape behavior, we used an estimate of feeding innovations based on an exhaustive survey of 30 years (1970–2000) of short note sections of 65 generalist ornithology journals covering 6 regions of the world done by L. Lefebvre and coworkers (Lefebvre et al. 1997; Nicolakakis and Lefebvre 2000). Because there may be more reports of feeding innovations available for intensely studied species, we estimated research effort by using the number of studies published since 1972 on each species as cited in the ISI Web of Science (http://apps.webofknowledge.com/) .

Prevalence of blood parasites

We used extensive data on prevalence of infection by *Haemoproteus*, *Leucocytozoon*, and *Plasmodium* relying on analyses of blood smears as reported in the literature combining information from Peirce (1981) and Scheuerlein and Ricklefs (2004). In addition, we used information fromMerilä et al. (1995), Sol et al. (2000), Merino et al. (2002), Navarro et al. (2004), and Møller AP and Merino S (unpublished data). Based on these data, we calculated for each bird species 3 proportions: The proportion of individuals infected by *Haemoproteus*, *Leucocytozoon*, and *Plasmodium*. Then we used these 3 proportions in the subsequent analyses. Finally, we recorded the number of individuals examined for each host species. This extensive dataset provided us with information for a total of 18 429 individual juvenile and adult hosts tested for the presence of blood parasites based on blood smears. We did not include nestlings because blood parasites are typically only present in the blood stream in juvenile and adult hosts (Valkiūnas 1991).

Habitat variables

We used 2 variables to quantify effects of habitat, relying on Møller and Garamszegi (2012). The first variable, which reflects habitat complexity, was scored as 0 for grassland, 1 for shrub, and 2 for trees for the main breeding habitat, assuming that more complex habitats result in greater exposure to vectors of blood parasites (Tella et al. 1999). The second variable, which reflects habitat generalism, was the total number of predefined breeding habitats for all species as reported by Cramp and Perrins (1977–1994). Cramp and Perrins (1977–1994) provide an extensive list of habitat categories predefined and used consistently throughout the handbook to describe breeding habitats. Therefore, a similar procedure was used to describe breeding habitats of all species. We skimmed the breeding habitat sections of all species for these habitat terms and simply added these to obtain a combined habitat score, for which larger values implied a higher degree of habitat generalism. The dataset is reported in Supplementary Table A1.

Migratory behavior and coloniality

Migration was scored as 0 for resident species, when the breeding and the winter distribution overlapped and 1 for migratory species. Coloniality was scored as 0 for solitary species and 1 for colonial species during the breeding season, when individuals defended small nest site territories and otherwise shared foraging habitats, as reported by Cramp and Perrins (1977–1994). The dataset is reported in Supplementary Table A1.

Statistical procedures

First, we determined the reliability and independence of our variables, testing whether the different components of escape behavior were repeatable and correlated with each other by calculating Pearson product-moment correlation coefficients. Similarly, we used Pearson product-moment correlation to test the relationship among all the variables that were included in our multivariate analysis. Flight initiation distance represents another well-studied component of antipredator behavior in the precapture phase of an interaction between a prey individual and a potential predator (Hediger 1934; Blumstein 2006). With the aim to determine whether flight initiation distance and escape behavior represent different axes of antipredator behavior, we tested whether these 2 variables were correlated. We estimated relative feeding innovation rate independent of research effort by using research effort as a covariate in the statistical models with feeding innovation as the response variable. All analyses were carried out for 85 avian host species by taking into account the statistical dependence of the data due to similarity caused by common phylogenetic descent.

We calculated repeatability of behavioral variables within species. We estimated consistency in behavior among individuals of the same species using the intraclass correlation coefficient. Repeatability is the variance between subjects relative to the total variance (e.g., between-species variance and residual variance due to within-species variance) (Garamszegi et al. 2009). This measurement was calculated following the study by Bell et al. (2009).

We calculated the mean value for each escape behavioral variable for each species and recorded the number of individuals on which this estimate was based. We treated all variables as continuous because binary variables can be treated as dummy variables (Sokal and Rohlf 2005) and intermediate states make biological sense. Tonic immobility, flight initiation distance, frequency of feeding innovations, research effort, and body mass were log transformed before analyses. Prevalence was arcsine-square-root transformed to satisfy requirements for normality.

With the aim to determine if the 6 behavioral variables could be entered in the analyses with no problems of collinearity, we conducted a principal component analysis on these variables using the correlation matrix and a varimax rotation. Eigen values for the first 3 components were 2.14, 1.09, and 0.97 accounting for 35.7%, 18.2%, and 16.2% of the variance, respectively.

Closely related species may have more similar behavior than species that are more distantly related due to common phylogenetic descent rather than convergence (Harvey and Pagel 1991). Therefore, analysis of data for multiple species requires statistical control for this phylogenetic component because it violates assumptions about independence of data. The phylogenetic relationship among host species was based on a composite supertree of most species of birds reported by Davis (2008) (Electronic Supplementary Material Figure A1). The phylogeny was constructed using the program Mesquite 2.75 (Maddison and Maddison 2011).

We analyzed the relationship between prevalence of the three genera of blood parasites as response variables and 6 escape behaviors, body mass, habitat variables, migration, and coloniality as predictors. Additionally, we tested for a correlation between behavioral variables and the frequency of feeding innovations where the logarithm of research effort was used as a covariate and the feeding innovation rates as a predictor for prevalence of blood parasites. We also tested for correlations between coloniality during the breading season and escape behavior where coloniality was treated as a continuous variable. All statistical analyses were carried out with the program R-2.15 (R Development Core Team 2011) using linear models while taking phylogenetic nonindependence of data points into account relying on phylogenetic generalized least-square models (PGLS) where the phylogenetic relationship was taken into account using the R packages *geiger* (Harmon et al. 2009) and *caper* (Orme et al. 2012). The strength and type of the phylogenetic signal in the residual matrix of the model can be accounted for by adjusting branch lengths (λ) (Freckleton et al. 2002). These transformations can be optimized to find the maximum likelihood transformation given the data and the model. To adjust for the heterogeneity in sampling effort across species (see Garamszegi and Møller 2010), we weighted each model by the underlying within-species sample size with the aim to make use of all the data relative to the precision of the estimates (Paradis 2011). Normality, homoscedasticity, and independence of residuals were verified.

Previous studies have criticized the use of Bonferroni correction of multiple statistical tests (Moran 2003; Nakagawa 2004; Garamszegi 2006; Garamszegi et al. 2009) because it could increase the risk of committing Type II errors (Nakagawa and Cuthill 2007). Nakagawa (2004) suggested that effect sizes and confidence intervals (CIs) reliably reveal the biological importance of results. Hence, we applied the effect size approach (based on Pearson's product-moment correlation coefficient r and its CI) to deal with the fact that we tested our predictions with a large number of variables that may raise issues about multiple testing that may severely affect P value–based interpretations in a null hypothesis testing framework. To calculate effect sizes, we used t of the particular effect in the model (where t = slope estimate/SE of the slope) and the corresponding df to calculate r and standard errors (based on r) to approximate the corresponding CIs (Nakagawa and Cuthill 2007). CIs (95%) are presented as lower/upper limits. For demonstrative purposes, we also present significance levels. In behavioral studies, the following benchmark is used for interpretations: $r \approx 0.1$ is a small effect, $r \approx 0.3$ is a intermediate effect, and $r \approx 0.5$ is a strong effect (Cohen 1988; Møller and Jennions 2002). The magnitude of effects in biological studies is typically intermediate accounting for 5–7% of the variance, thus being equivalent of effect sizes of 0.22–0.26 (Møller and Jennions 2002).

Ethical note

There was no animal experimentation involved in this study because all data were derived from a literature survey. Hence the study complied with all legal ethical requirements in the countries of residence of the 3 authors.

results

Mean values, standard errors (SE), and range of escape behavior variables are reported in Table 1. We also report repeatabilities based on multiple measures among individuals within species that are consistent within each variable. None of the components

Table 1
Summary statistics for escape behavior among species of birds and within-species repeatability (*R*)

Variable	Mean	SE	Range	CV	*F*	*R* (SE)
Wriggle	1.067	0.042	0–3	215	13.94	0.220 (0.006)
Biting	0.292	0.034	0–1	628	54.83	0.540 (0.008)
Feather loss	0.135	0.023	0–1	942	31.18	0.395 (0.007)
Tonic immobility (s)	11.631	0.495	0–30	231	5.65	0.092 (0.003)
Fear scream	0.145	0.019	0–1	723	12.65	0.202 (0.005)
Alarm call	0.248	0.024	0–1	537	14.71	0.230 (0.006)

CV indicates coefficient of variation. df for the *F* values are 97, 4405.

of escape behavior were correlated across species with flight initiation distance with a magnitude that hardly reached intermediate effect size in models that also included body mass as a predictor variable (wriggle: $r = 0.075$, 95% CI: −0.140/0.283, $P = 0.46$; biting: $r = 0.199$, 95% CI: 0.014/0.395, $P = 0.006$; feather loss: $r = 0.048$, 95% CI: −0.166/0.258, $P = 0.14$; tonic immobility: $r = 0.042$, 95% CI: −0.172/0.253, $P = 0.67$; alarm call: $r = 0.094$, 95% CI: −0.121/0.301, $P = 0.35$; fear scream: $r = 0.087$, 95% CI: −0.128/0.294, $P = 0.39$). Therefore, escape behavior mostly represented different components of antipredator behavior other than flight initiation distance.

The escape behavior variables were only weakly correlated with each other (the largest Pearson product-moment correlation coefficient was $r = 0.35$ between wriggle and fear scream; all others had $|r| < 0.26$). Analyses based on principal component analysis of behavioral variables provided qualitatively similar results, and, therefore, we only present the analyses based on the individual behavioral variables for transparency. Habitat, migration, coloniality, and 6 behavioral variables were also weakly correlated with each other (the 2 largest Pearson product-moment correlation coefficients were $r = 0.39$ between feather loss and habitat complexity and $r = 0.38$ between fear scream and habitat complexity; all other had $|r| < 0.29$) implying that both behavioral and habitat variables could be entered in the analyses without serious problems of collinearity (Harmon et al. 2008). We calculated the correlation among *Haemoproteus*, *Plasmodium*, and *Leucocytozoon*. The 3 genera were weakly correlated with each other (the largest Pearson product-moment correlation coefficient was $r = 0.36$ between *Plasmodium* and *Haemoproteus*; all others had $|r| < 0.09$); thus, we can reasonably assume that they are independent.

Fear scream and habitat generalism were correlated with prevalence of *Haemoproteus* with an intermediate effect size accompanied by a rather broad and positive range of CIs. The biological interpretation of this effect is that species with a higher prevalence of *Haemoproteus* likely occupy more different habitats than species with a lower prevalence of infection by *Haemoproteus* (Table 2). We detected an effect size that showed an intermediate relationship between fear scream and prevalence of *Haemoproteus*. Therefore, from the current data, we concluded that species with high prevalence of *Haemoproteus* are very likely to have a higher fraction of individuals giving fear screams than species with lower prevalence (Table 2; Figure 1). Components of escape behavior and habitat generalism were correlated with prevalence of infection by *Leucocytozoon* with an effect size and CIs that imply a positive association: Species with a higher prevalence of *Leucocytozoon* were found in several different habitats than species with a lower prevalence of *Leucocytozoon* (Table 2; Figure 2). The intensity of wriggle showed intermediate effects in relation to prevalence of *Leucocytozoon*. Accordingly, species with high prevalence of *Leucocytozoon* wriggled more while being held in a hand (Table 2). The effect size range for the frequency of feather loss indicated that it is likely to decrease with increasing prevalence, as expected (Table 2). Species with a higher prevalence of infection by *Plasmodium* were found in several different habitats than species with a lower prevalence of *Plasmodium* (Table 2). We found an intermediate effect size for the relationship between *Plasmodium* and habitat complexity with a confidence range showing that species with a higher prevalence of *Plasmodium* were found in habitats with a greater complexity (Table 2). None of the 6 escape behavioral variables were correlated with the prevalence of infection by *Plasmodium* with a magnitude and CI that could have biological relevance (Table 2). However, there was a positive correlation between log body mass and prevalence of infection by *Haemoproteus*, *Leucocytozoon*, and *Plasmodium*, in the expected direction (Table 2).

The relative frequency of feeding innovations was correlated by intermediate effect sizes with the frequency of biting and tonic immobility, with a positive and negative association, respectively (Table 3). However, the frequency of feeding innovations is unlikely to be an important predictor of blood parasite prevalence in models that included body mass, escape behavior as predictors, and research effort as a covariate (*Leucocytozoon*: $r = -0.024$, 95% CI: −0.237/0.188, $t = -0.23$, $P = 0.82$; *Haemoproteus*: $r = -0.060$, 95% CI: −0.270/0.153, $t = -0.56$, $P = 0.58$; *Plasmodium*: $r = -0.165$, 95% CI: −0.367/0.047, $t = -1.55$, $P = 0.13$). Hence, we did not include feeding innovation in the multivariate analyses due to feeding innovation not being an important predictor of prevalence of blood parasites.

Coloniality was positively correlated with an intermediate effect size with tonic immobility (Table 4), with colonial species taking less time to right themselves and fly away than solitary species.

In most of our statistical analyses, we found a value of lambda (λ) larger than 0, but smaller than 0.085, which indicates that these correlations are hardly influenced by the phylogenetic relationships among the bird species analyzed in this study.

Predictors including escape behavior and body mass in the same statistical model on prevalence of blood parasites resulted in effect sizes for feeding innovations that are far from being biologically important (Supplementary Table A2). Hence escape behavior and feeding innovation rate seemed to be independent of each other.

Discussion

In this study of escape behavior of 85 species of birds, we found effect sizes that suggest 2 considerable, positive relationships between antipredator behavior and prevalence of blood parasites. These relationships could be estimated with a CI that covered small to intermediate effects that we interpret as biologically meaningful (*sensu* Møller and Jennions 2002). Therefore, we conclude that 1) bird species with a higher species-specific prevalence

Table 2

Infection by *Haemoproteus*, *Leucocytozoon*, and *Plasmodium* in relation to escape behavior, migration, coloniality, habitat complexity, and habitat generalism (number of breeding habitats)

Factor	Value	SE	*t*	Effect size	95% CIs		*P*
					Lower	Upper	
Haemoproteus							
Alarm call	0.091	0.065	1.38	0.155	−0.071	0.366	0.170
Biting	0.077	0.048	1.58	0.177	−0.048	0.385	0.117
Wriggle	−0.065	0.031	−1.65	0.184	−0.041	0.392	0.091
Feather loss	0.095	0.067	1.41	0.158	−0.068	0.368	0.161
Fear scream	**0.120**	**0.059**	**2.37**	**0.261**	**0.039**	**0.458**	**0.016**
log Tonic immobility (s)	0.038	0.040	0.95	0.107	−0.119	0.323	0.341
log Mass (g)	**0.136**	**0.057**	**2.34**	**0.257**	**0.035**	**0.454**	**0.021**
Habitat complexity	−0.004	0.033	−0.13	0.015	−0.209	0.238	0.891
Habitat generalism	**0.014**	**0.006**	**2.34**	**0.257**	**0.035**	**0.454**	**0.022**
Migration	0.071	0.064	1.11	0.125	−0.102	0.339	0.269
Coloniality	−0.097	0.099	−0.97	0.109	−0.118	0.325	0.331
λ = 0.021, residual SE = 0.083, df = 73							
Leucocytozoon							
Alarm call	0.047	0.051	0.93	0.105	−0.121	0.321	0.354
Biting	0.042	0.038	1.11	0.125	−0.102	0.339	0.267
Wriggle	**0.073**	**0.024**	**2.96**	**0.319**	**0.102**	**0.507**	**0.004**
Feather loss	**−0.081**	**0.033**	**−2.12**	**0.234**	**0.011**	**0.435**	**0.041**
Fear scream	0.014	0.062	0.23	0.026	−0.199	0.248	0.813
log Tonic immobility (s)	0.016	0.031	0.51	0.058	−0.168	0.278	0.605
log Mass (g)	**0.127**	**0.043**	**2.94**	**0.317**	**0.100**	**0.505**	**0.004**
Habitat complexity	0.036	0.025	1.41	0.158	−0.068	0.368	0.161
Habitat generalism	**0.014**	**0.004**	**3.07**	**0.330**	**0.114**	**0.515**	**0.003**
Migration	−0.061	0.049	1.24	0.139	−0.087	0.352	0.216
Coloniality	0.031	0.077	0.40	0.045	−0.181	0.266	0.687
λ = 0.059, residual SE = 0.063, df = 73							
Plasmodium							
Alarm call	−0.089	0.074	−1.20	−0.135	−0.348	0.091	0.231
Biting	0.032	0.028	1.13	0.127	−0.099	0.341	0.260
Wriggle	−0.027	0.018	−1.46	−0.164	−0.374	0.062	0.147
Feather loss	0.016	0.039	0.41	0.046	−0.179	0.267	0.682
Fear scream	0.007	0.047	0.16	0.018	−0.206	0.241	0.869
log Tonic immobility (s)	0.004	0.023	0.19	0.021	−0.203	0.243	0.849
log Mass (g)	**0.065**	**0.032**	**1.99**	**0.221**	**0.002**	**0.424**	**0.049**
Habitat complexity	**0.072**	**0.023**	**2.01**	**0.223**	**0.001**	**0.425**	**0.041**
Habitat generalism	**0.067**	**0.027**	**2.10**	**0.232**	**0.008**	**0.433**	**0.038**
Migration	−0.001	0.037	−0.01	−0.001	−0.224	0.223	0.999
Coloniality	−0.019	0.058	−0.33	−0.037	−0.258	0.188	0.735
λ = 0.081, residual SE = 0.048, df = 73							

Test statistics refer to linear estimates and their standard errors, and the associated *P* values in phylogenetic analyses weighted by sample size. Effect sizes (*r*) are Pearson product-moment correlation coefficients with their 95% CIs. Effect sizes differing from zero are shown in bold.
Effect size conventions: $r = 0.10$, small effect; $r = 0.30$, medium effect; and $r = 0.50$, large effect (Cohen 1988).

of *Haemoproteus* had a higher fraction of individuals emitting fear scream than species with fewer infected individuals; and 2) bird species infected by *Leucocytozoon* tended to wriggle more when held in a hand. In contrast, we failed to detect strong relationships between prevalence of infection by *Plasmodium* and escape behavior. The effect sizes and the associated CIs for correlations between blood parasite prevalence and habitat generalism indicated that prevalence of the 3 genera of blood parasites was higher in species that were habitat generalists. Additionally, species with greater habitat complexity showed higher prevalence of *Plasmodium*. These associations were unlikely to be caused by a greater extent of habitat exploration as reflected by the relative frequency of feeding innovations although innovations were more frequent in species that were commonly biting and showed high rates of tonic immobility. Furthermore, coloniality was correlated with tonic immobility. Below, we discuss the biological meaning of these effects.

Escape behavior constitutes a suite of antipredator behavior directed at escape from a predator once captured (Møller et al. 2011). Bird species with more individuals emitting fear screams and struggling more when held in a hand showed higher prevalence of *Haemoproteus* and *Leucocytozoon*. These components of escape behavior are related to susceptibility to predation. Högstedt (1983) and

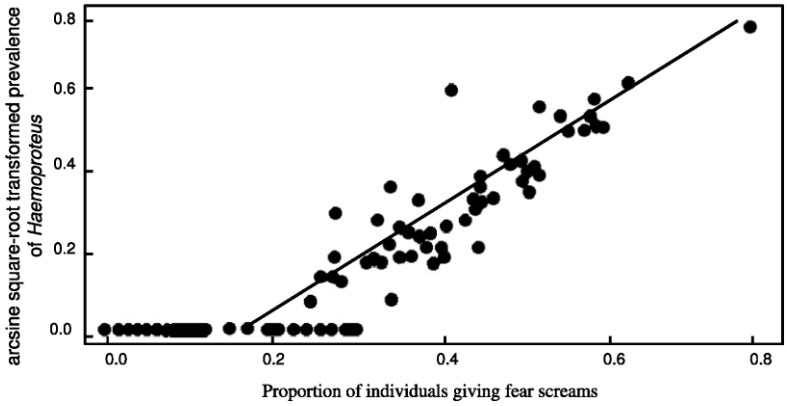

Figure 1
Proportion of individuals giving a fear scream in different species of birds in relation to prevalence of *Haemoproteus*. The lines are the linear regression lines.

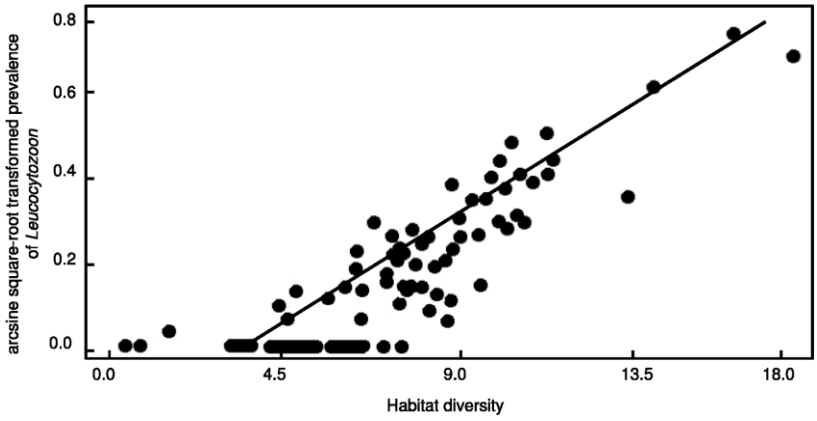

Figure 2
Breeding habitat diversity in different species of birds in relation to prevalence of *Leucocytozoon*. The line is the linear regression line.

Møller et al. (2010) found that the probability to attract secondary predators increased if birds emitted fear screams when handled. Furthermore, wriggle score was positively related to susceptibility to predation by cats and hawks (Møller et al. 2011), with birds wriggling more having higher probability of escape. These results suggest that species with a higher intensity of escape behavior have higher prevalence of blood parasites, as predicted. Additionally, it has been shown that individuals faced with predators have a higher probability of infection with blood parasites than individuals encountering an innocuous control like a pigeon (Navarro et al. 2004). Navarro et al. (2004) found that an increase in predation risk resulted in a higher probability of infection, and we found a correlation between escape behavior and prevalence of blood parasites. Thus, we can deduce that species with increased prevalence will also exhibit higher levels of escape behavior and risk of predation.

Infection by *Plasmodium* was not correlated with escape behavior in contrast to what was observed for the other 2 genera of blood parasites. This difference among genera of avian blood parasites could be due to Hemosporidian life cycles differing among genera (Valkiūnas 2005). *Plasmodium* can cause host death, and *Plasmodium* is responsible for particularly severe outbreaks of malaria in domestic birds (Garnham 1980; Huchzermeyer 1993). Therefore, *Plasmodium* may eliminate a larger fraction of infected individuals before they are caught and scored with respect to escape behavior, and this may explain the results of our study concerning *Plasmodium*. In contrast,Møller and Nielsen (2007) found that infection with *Plasmodium* was associated with elevated susceptibility to predation by 2 species of hawks. These 2 effects may result in an unreliable prevalence of *Plasmodium* due to the elimination of infected individuals by predators. Bird species with higher prevalence of *Plasmodium*, *Haemoproteus*, and *Leucocytozoon* were found in a larger number of breeding habitats than species with lower prevalence. This result is consistent with the hypothesis that habitat generalists more readily encounter vectors infected by blood parasites than habitat specialists.Møller and Garamszegi (2012) have shown that individuals with a greater variance in antipredator defense as reflected by flight initiation distance exploit a greater diversity of breeding habitats. Thus, species composed of individuals with bolder behavior and higher degrees of habitat generalism may have a higher prevalence of blood parasites. Moreover, species inhabiting habitats with a greater complexity showed a higher prevalence of *Plasmodium*. However, we should be cautious when interpreting these results because the mortality caused by hemosporidians may lead to undersampling of infected individuals in field studies (Valkiūnas 1993). Thus, there are reasons to assume that a greater diversity of habitats results in more encounters with vectors of blood parasites. However, we cannot determine from our correlative analyses whether a higher prevalence in a given host species

Table 3
Frequency of feeding innovations in relation to escape behavior

Behavioral variable	Value	SE	*t*	Effect size	95% CIs		*P*
					Lower	Upper	
log Research effort	**0.967**	**0.022**	**4.39**	**0.454**	**0.255**	**0.617**	**<0.001**
Alarm call	0.142	0.077	1.96	0.222	−0.004	0.426	0.053
Biting	**0.146**	**0.054**	**2.69**	**0.298**	**0.078**	**0.491**	**0.008**
Wriggle	−0.005	0.035	−0.01	−0001	−0.226	0.224	0.998
Feather loss	0.101	0.070	1.44	0.165	−0.063	0.376	0.152
Fear scream	−0.008	0.084	−0.09	−0.011	−0.235	0.215	0.922
log Tonic immobility (s)	**−2.244**	**0.040**	**−6.08**	**−0.577**	**−0.710**	**−0.404**	**<0.001**
log Mass (g)	0.040	0.077	0.52	0.060	−0.167	0.282	0.602
λ = 0.868, residual SE = 0.154, df = 76							

Test statistics refer to linear estimates and their standard errors, and the associated effect sizes (*r*) are Pearson product-moment correlation coefficients with their 95% CIs. Effect sizes differing from zero are shown in bold.
Effect size conventions: *r* = 0.10, small effect; *r* = 0.30, medium effect; and *r* = 0.50, large effect (Cohen 1988).

Table 4
Coloniality in relation to escape behavior and blood parasite prevalence

Behavioral variable	Value	SE	*t*	Effect size	95% CIs		*P*
					Lower	Upper	
Biting	0.053	0.056	0.94	0.108	−0.123	0.328	0.347
Alarm call	−0.095	0.077	−1.23	0.141	−0.091	0.358	0.221
Fear scream	0.004	0.085	0.04	0.004	−0.224	0.232	0.961
Feather loss	−0.003	0.068	−0.04	0.004	−0.224	0.232	0.961
log Tonic immobility (s)	**−0.102**	**0.042**	**−2.41**	**0.269**	**0.043**	**0.468**	**0.018**
log Mass (g)	0.006	0.066	0.09	0.010	−0.219	0.238	0.928
Wriggle	−0.065	0.040	−1.62	0.185	−0.045	0.396	0.107
λ = 0.100, residual SE = 0.098, df = 74							

Test statistics refer to linear estimates and their standard errors, and the associated effect sizes (*r*) are Pearson product-moment correlation coefficients with their 95% CIs. Effect sizes differing from zero are shown in bold.
Effect size conventions: *r* = 0.10, small effect; *r* = 0.30, medium effect; and *r* = 0.50, large effect (Cohen 1988).

is due to a greater intensity of escape behavior resulting in exposure to different habitats or whether species with high prevalence frequent many different habitats because they display a higher intensity of antipredator behavior. We cannot propose any particular causal scenario based on our results, and further experimental approaches would be needed to clarify the causal relationship among the 3 correlated variables.

We have shown that species with a higher prevalence of blood parasites more often gave fear screams and struggled more when held in a hand. It is possible that such escape behavior causes a higher encounter rate with vectors resulting in higher prevalence of blood parasites. It would be interesting to know if prevalence of blood parasites affects escape behavior, or escape behavior affects prevalence. Many blood parasites rely on a change in behavior of their hosts in order to complete their life cycle (Webster et al. 1994). No studies have so far suggested that *Plasmodium*, *Haemoproteus*, and *Leucocytozoon* provoke changes in escape behavior although Dunn et al. (2011) suggested that *Plasmodium* and *Leucocytozoon* could have a broader effect on behavior than previously assumed. Habitat generalists could have a higher probability of encountering vectors infected with blood parasites simply because they range more widely and hence experience a greater risk of infection. This hypothesis should be tested experimentally with the aim to clarify the direction of the causal relationship between these traits.

We explicitly tested whether the relative rate of feeding innovations, a trait that also has been found to be positively correlated with blood parasitism (Garamszegi et al. 2007), predicted escape behavior. We found some evidence consistent with this hypothesis. Indeed both the proportion of individuals biting and the duration of tonic immobility were correlated with feeding innovations. These findings suggest that the correlation between escape behavior and the relative rate of feeding innovations could have a cognitive basis as shown by correlations with relative brain size (Lefebvre et al. 1997; Nicolakakis and Lefebvre 2000). However, a considerable number of behavioral variables vary independently of innovation, and thus, the patterns we found in relation to parasitism cannot be fully attributed to interspecific differences in cognition mediating escape behavior.

It has been hypothesized that migratory species would show higher prevalence of blood parasites due to such species visiting a larger number of habitats and being exposed to a more diverse parasite fauna (Bennett and Fallis 1960; Greiner et al. 1975; Waldenström et al. 2002; Møller and Szép 2011). Accordingly, immune function of migratory species appears to be stronger than in residents (Møller et al. 2004; Møller and Erritzøe 2008; Møller and Szép 2011). However, we failed to detect any relationship between migratory habits and prevalence of blood parasites.

There is generally a positive correlation between group size and parasitism (Alexander 1974; Rifkin et al. 2012). Colonial species should have higher probabilities of getting infected by blood parasites due to efficient horizontal transmission. However, none of the 3 genera of blood parasite was related to coloniality during

the breeding season. However, we found a negative correlation between coloniality and escape behavior, with colonial species having shorter tonic immobility than solitary species. These results agree with studies suggesting that colonial breeders may experience higher predation risks than solitary species (Marchant and Higgins 1993; Dall and Griffith 2014). This suggests that variation in tonic immobility can be interpreted as differences in boldness toward predators (Edelaar et al. 2012).

This study has a number of implications for future studies of specific host-blood parasite model systems. It would be interesting to test whether an infected individual shows a higher intensity of escape behavior. It would also be interesting to test whether bird species show an increase in intensity of escape behavior when experimentally infected with blood parasites, and whether medication with antimalarial drugs such as primaquine causes a reduction in the intensity of escape behavior. Although previous studies have demonstrated that behavioral traits show substantial spatial variation within species (Møller 2008b), there is still a high degree of repeatability in behavior among habitats (Møller 2008b). Hence, it would be interesting to test whether the difference in prevalence of hemosporidians between habitats is related to the difference in host behavior between habitats. It would also be interesting to test whether different parasite lineages have a similar effect on escape behavior or whether they differ in effect as suggested by the present study. Finally, it would be interesting to test experimentally if species that have larger home ranges and visit a greater diversity of habitats accumulate more blood parasite lineages.

Supplementary Material

Supplementary material can be found at http://www.beheco.oxfordjournals.org/.

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References

Alexander RD. 1974. The evolution of social behaviour. Annu Rev Ecol Syst. 5:325–383

Barber I, Dingemanse NJ. 2010. Parasitism and the evolutionary ecology of animal personality. Philos Trans R Soc Lond B Biol Sci. 365:4077–4088.

Bell AM, Hankison SJ, Laskowski KL. 2009. The repeatability of behaviour: a meta-analysis. Anim Behav. 77:771–783.

Belliure J, Sorci G, Møller AP, Clobert J. 2000. Dispersal distances predict subspecies richness in birds. J Evol Biol. 13:480–487.

Bennett GF, Fallis AM. 1960. Geiger: blood parasites of birds in algonquin park, Canada, and a discussion of their transmission. Can J Vet Res. 38:261–273.

Blumstein DT. 2006. Developing an evolutionary ecology of fear: how life history and natural history traits affect disturbance tolerance in birds. Anim Behav. 71:389–399.

Boissy A. 1995. Fear and fearfulness in animals. Q Rev Biol. 70:165–191.

Boyer N, Réale D, Marmet J, Pisanu B, Chapuis JL. 2010. Personality, space use and tick load in an introduced population of Siberian chipmunks Tamias sibiricus. J Anim Ecol. 79:538–547.

Breman JG. 2001. The ears of the hippopotamus: manifestations, determinants, and estimates of the malaria burden. Am J Trop Med Hyg. 64:1–11.

Charnov LE, Krebs RJ. 1975. The evolution of alarm calls: altruism or manipulation? Am Nat. 109:107–112.

Clinchy M, Zanette L, Boonstra R, Wingfield JC, Smith JNM. 2004. Balancing food and predator pressure induces chronic stress in songbirds. Proc R Soc Lond Ser B Biol Sci. 271:2473–2479.

Cohen J. 1988. Statistical power analysis for the behavioural sciences. Hillsdale (MI): Lawrence Erlbaum Associates.

Cornet S, Nicot A, Rivero A, Gandon S. 2013. Malaria infection increases bird attractiveness to uninfected mosquitoes. Ecol Lett. 16:323–329.

Cramp S, Perrins CM. 1977–1994. Handbook of the birds of Europe, the Middle East and NorthAfrica: the birds of the Western Paleartic. Oxford (UK): Oxford University Press.

Dall SRX, Griffith SC. 2014. An empiricist guide to animal personality variation in ecology and evolution. Front Ecol Evol. doi:10.3389/fevo.2014.00003.

Dammhahn M, Almeling L. 2012. Is risk taking during foraging a personality trait? A field test for cross-context consistency in boldness. Anim Behav. 84:1131–1139.

Davis KE. 2008. Reweaving the tapestry: a supertree of birds [dissertation]. [Glasgow (UK)]: University of Glasgow.

Dawson RD, Bortolotti GR. 2000. Effects of hematozoan parasites on condition and return rates of American kestrels. Auk. 117:373–380.

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

Dunn JC, Cole EF, Quinn JL. 2011. Personality and parasites: sex-dependent associations between avian malaria infection and multiple behavioural traits. Behav Ecol Sociobiol. 65:1459–1471.

Edelaar P, Serrano D, Carrete M, Blas J, Potti J, Tella JL. 2012. Tonic immobility is a measure of boldness toward predators: an application of Bayesian structural equation modelling. Behav Ecol. 23:619–626.

Endler JA. 1986. Natural selection in the wild. Princeton (NJ): Princeton University Press.

Forkman B, Boissy A, Meunier-Salaün MC, Canali E, Jones RB. 2007. A critical review of fear tests used on cattle, pigs, sheep, poultry and horses. Physiol Behav. 92:340–374.

Freckleton RP, Harvey PH, Pagel M. 2002. Phylogenetic analysis and comparative data: a test and review of evidence. Am Nat. 160:712–726.

Garamszegi LZ. 2006. Comparing effect sizes across variables: generalization without the need for Bonferroni correction. Behav Ecol. 17:682–687.

Garamszegi LZ. 2011. Climate change increases the risk of malaria in birds. Global Change Biol. 17:1751–1759.

Garamszegi LZ, Calhim S, Dochtermann N, Hegyi G, Hurd PL, Jorgensen C, Kutsukake N, Lajeunesse MJ, Pollard K a., Schielzeth H, et al. 2009. Changing philosophies and tools for statistical inferences in behavioural ecology. Behav Ecol. 20:1363–1375.

Garamszegi LZ, Erritzøe J, Møller A. 2007. Feeding innovations and parasitism in birds. Biol J Linn Soc. 90:441–455.

Garamszegi LZ, Møller AP. 2010. Effects of sample size and intraspecific variation in phylogenetic comparative studies: a meta-analytic review. Biol Rew. 85:797–805.

Garnham PCC. 1980. Malaria in its various vertebrate hosts. In: Kreier JP, editor. Miscellaneous malaria: epidemiology, chemotherapy, morphology, and metabolism Vol. 1. New York: Academic Press. p. 95–144.

Greiner EC, Bennett GF, White EM, Coombs RF. 1975. Distribution of the avian hematozoa of North America. Can J Vet Res. 53:1762–1787.

Harmon L, Weir J, Brock C, Glor R, Challenger W, Hunt G. 2009. R: a language and environment for statistical computing. Vienna (Austria): R Foundation for Statistical Computing. R Found Stat Comput. 1.

Harmon LJ, Weir JT, Brock CD, Glor RE, Challenger W. 2008. GEIGER: investigating evolutionary radiations. Bioinformatics. 24:129–131.

Harvey PH, Pagel MD. 1991. The comparative method in evolutionary biology. Oxford: Oxford University Press.

Hediger H. 1934. Zur Biologie and Psychologie der Flucht bei Tieren. Biologisches Zentralblatt. 54:21–40.

Hellgren O, Pérez-Tris J, Bensch S. 2009. A jack-of-all-trades and still a master of some: prevalence and host range in avian malaria and related blood parasites. Ecology. 90:2840–2849.
Hoagland H. 1928. On the mechanism of tonic immobility in vertebrates. J Gen Physiol. 11:715–741.
Högstedt G. 1983. Adaptation unto death: function of fear screams. Am Nat. 121:562–574.
Huchzermeyer FW. 1993. A host-parasite list of the haematozoa of domestic poultry in sub-Saharan Africa and the isolation of Plasmodium durae Herman from turkeys and francolins in South Africa. Onderstepoort J Vet Res. 60:15–21.
Ibáñez-Álamo JD, Soler M. 2010. Does urbatization affect selective pressures and life-history strategies in the common blackbird (*Turdus merula* L.)? Biol J Linn Soc. 101:759–766.
Jones RB. 1986. The tonic immobility reaction of the domestic fowl: a review. World Poultry Sci J. 42:82–96.
Lachish S, Knowles SC, Alves R, Wood MJ, Sheldon BC. 2011. Fitness effects of endemic malaria infections in a wild bird population: the importance of ecological structure. J Anim Ecol. 80:1196–1206.
Lefebvre L, Whittle P, Lascaris E. 1997. Feeding innovations and forebrain size in birds. Anim Behav. 53:549–560.
Levine ND. 1988. The Protozoan Phylum Apicomplexa. Boca Raton (FL): CRC Press.
MacDougall-Shackleton EA, Derryberry EP, Hahn TP. 2002. Nonlocal male mountain white-crowned sparrows have lower paternity and higher parasite loads than males singing local dialect. Behav Ecol. 13:682–689.
Maddison WP, Maddison DR. 2011. Mesquite: a modular system for evolutionary analysis. Version 2.75. Sunderland (MA): Sinauer.
Marchant S, Higgins PJ. 1993. Handbook of Australian, New Zealand and Antartic birds Vol. 2: Raptors to Lapwings. Melbourne (Australia): Oxford University.
Martinsen ES, Perkins SL, Schall JJ. 2008. A three-genome phylogeny of malaria parasites (Plasmodium and closely related genera): evolution of life-history traits and host switches. Mol Phylogenet Evol. 47:261–273.
Marzal A, de Lope F, Navarro C, Møller AP. 2005. Malarial parasites decrease reproductive success: an experimental study in a passerine bird. Oecologia. 142:541–545.
Merilä J, Björklund M, Bennett GF. 1995. Geographic and individual variation in haematozoan infections in the greenfinch, *Carduelis chloris*. Can J Zool. 73:1798–1804.
Merino S, Martinez J, Moller AP, Barbosa A, de Lope F, Rodriguez-Caabeiro F. 2002. Blood stress protein levels in relation to sex and parasitism of barn swallows (*Hirundo rustica*). Ecoscience. 9:300–305.
Merino S, Moreno J, Jose J, Arriero E. 2000. Are avian blood parasites pathogenic in the wild? A medication experiment in blue tits (*Parus caeruleus*). Proc R Soc Lond B Biol Sci. 9:2507–2510.
Møller AP. 2008a. Flight distance and population trends in European breeding birds. Behav Ecol. 19:1095–1102.
Møller AP. 2008b. Flight distance of urban birds, predation, and selection for urban life. Behav. Ecol Sociobiol. 63:63–75.
Møller AP, Christiansen SS, Mousseau TA. 2011. Sexual signals, risk of predation and escape behaviour. Behav Ecol. 22:800–807.
Møller AP, de Lope F, Saino N. 2004. Parasitism, immunity, and arrival date in a migratory bird, the barn swallow. Ecology. 85:206–219.
Møller AP, Erritzøe J. 2008. Host immune defence and migration in birds. Ecol Lett. 12:945–953.
Møller AP, Garamszegi LZ. 2012. Between individual variation in risk-taking behaviour and its life history consequences. Behav Ecol. 23:843–853.
Møller AP, Ibáñez-Álamo JD. 2012. Escape behaviour of birds provides evidence of predation being involved in urbanization. Anim Behav. 84:341–348.
Møller A, Jennions M. 2002. How much variance can be explained by ecologists and evolutionary biologists? Oecologia. 132:492–500.
Møller AP, Nielsen JT. 2007. Malaria and risk of predation: a comparative study of birds. Ecology. 88:871–881.
Møller AP, Nielsen JT. 2010. Fear screams and adaptation to avoid imminent death: effects of genetic variation and predation. Ethol Ecol Evol. 22:183–202.
Møller AP, Nielsen JT, Erritzøe J. 2006. Losing the last feather: feather loss as an antipredator adaptation in birds. Behav Ecol. 17:1046–1056.
Møller AP, Nielsen JT, Erritzøe J. 2010. Causes of interspecific variation in susceptibility to cat predation on birds. Chinese Birds. 1:1–15.
Møller AP, Szép T. 2011. The role of parasites in ecology and evolution of migration and migratory connectivity. J Ornithol. 152:141–150.
Moore J. 2002. Parasites and the behaviour of animals. Oxford: Oxford University Press.
Moran MD. 2003. Arguments for rejecting the sequential Bonferroni in ecological studies. Oikos. 102:403–405.
Mostowy R, Engelstädter J. 2011. The impact of environmental change on host-parasite coevolutionary dynamics. Proc Biol Sci. 278:2283–2292.
Nakagawa S. 2004. A farewell to Bonferroni: the problems of low statistical power and publication bias. Behav Ecol. 15:1044–1045.
Nakagawa S, Cuthill IC. 2007. Effect size, confidence interval and statistical significance: a practical guide for biologists. Biol Rev Camb Philos Soc. 82:591–605.
Navarro C, de Lope F, Marzal A, Møller AP. 2004. Predation risk, host immune response, and parasitism. Behav Ecol. 15:629–635.
Nicolakakis N, Lefebvre L. 2000. Forebrian size and innovation rate in European birds: feeding, nesting and confounding variables. Behaviour. 137:1415–1429.
Orme CDL, Freckleton RP, Thomas GH, Petzoldt T, Fritz SA, Isaac NJB. 2012. CAPER: comparative analyses of phylogenetics and evolution in R. Methods Ecol. Evol. 3:145–151.
Paaijmans KP, Read AF, Thomas MB. 2009. Understanding the link between malaria risk and climate. Proc Natl Acad Sci USA. 106:13844–13849.
Palinauskas V, Valkiūnas G, Bolshakov CV, Bensch S. 2008. Plasmodium relictum (lineage P-SGS1): effects on experimentally infected passerine birds. Exp Parasitol. 120:372–380.
Paradis E. 2011. Analysis of phylogenetics and evolution with R. 2nd ed. Berlin (Germany): Springer.
Patz JA, Olson SH. 2006. Malaria risk and temperature: influences from global climate change and local land use practices. Proc Natl Acad Sci USA. 103:5635–5636.
Peirce MA. 1981. Distribution and host-parasite check-list of the haematozoa of birds in Western Europe. J Nat Hist. 15:419–458.
Platzen D, Magrath RD. 2004. Parental alarm calls suppress nestling vocalization. Proc Biol Sci. 271:1271–1276.
R Development Core Team. 2011. R: a language and environment for statistical computing. Vienna (Austria): R Foundation for Statistical Computing.
Rifkin JL, Nunn CL, Garamszegi LZ. 2012. Do animals living in larger groups experience greater parasitism? A meta-analysis. Am Nat. 180:70–82.
Roulin A, Wink M. 2004. Predator-prey relationships and the evolution of colour polymorphism: a comparative analysis in diurnal raptors. Biol J Linn Soc. 81:565–578.
Scheuerlein A, Ricklefs RE. 2004. Prevalence of blood parasites in European passeriform birds. Proc Biol Sci. 271:1363–1370.
Sims V, Evans KL, Newson SE, Tratalos J, Gaston KJ. 2008. Avian assemblage structure and domestic cat densities in urban environments. Divers Distrib. 14:387–399.
Sokal RR, Rohlf FJ. 2005. Biometry. New York: Freeman.
Sol D, Jovani R, Torres J. 2000. Geographical variation in blood parasites in feral pigeons : the role of vectors. Ecography. 23:307–314.
Sol D, Lefebvre L, Rodríguez-Teijeiro JD. 2005. Brain size, innovative propensity and migratory behaviour in temperate Palaearctic birds. Proc Biol Sci. 272:1433–1441.
Sorace A, Gustin M. 2009. Distribution of generalist and specialist predators along urban gradients. Landscape Urban Plan. 90:111–118.
Tella JL, Blanco G, Forero MG, Gajón A, Donázar JA, Hiraldo F. 1999. Habitat, world geographic range, and embryonic development of hosts explain the prevalence of avian hematozoa at small spatial and phylogenetic scales. Proc Natl Acad Sci USA. 96:1785–1789.
Tomás G, Merino S, Moreno J, Morales J. 2007. Consequences of nest reuse for parasite burden and female health and condition in blue tits, *Cyanistes caeruleus*. Anim Behav. 73:805–814.
Valkiūnas G. 1987. Blood parasites of birds of White Sea- Baltic direction of migration 4. Ecological aspect. Parazitologiya (St. Petersburg). 21:537–544 (in Russian).
Valkiūnas G. 1991. On the pathogenic influence of haemoproteids (Haemosporidia: Haemoproteidae) on wild birds in the field conditions. Parazitologiya (St. Petersburg). 25:404–411 (in Russian).
Valkiūnas G. 1993. Pathogenic influence of haemosporidians and trypanosomes on wild birds in the field conditions: facts and hypotheses. Ekologija (Vilnius). 1:47–60.

Valkiūnas G. 2005. Avian malaria parasites and other Haemosporidia. Boca Raton (FL): CRC Press.

Valkiūnas G, Tadas Z, Shapoval AP, Iezhova TA. 2006. Effect of Haemoproteus belopolskyi (Haemosporida : Haemoproteidae) on body mass of the Blackcap *Sylvia atricapilla*. Am Soc Parasitol. 92:1123–1125.

Waldenström J, Bensch S, Kiboi S, Hasselquist D, Ottosson U. 2002. Cross-species infection of blood parasites between resident and migratory songbirds in Africa. Mol Ecol. 11:1545–1554.

Webster JP, Brunton CFA, Macdonald DW. 1994. Effect of Toxoplasma-Gondii upon neophobic behaviour in wild brown rats, *Rattus norvegicus*. Parasitol. 109:37–43.

Wilson DS, Coleman K, Clark AB, Biederman L. 1993. Shy-bold continuum in pumpkinseed sunfish (*Lepomis gibbosus*): an ecological study of a psychological trait. J Comp Psychol. 107:250–260.

Wood MJ, Cosgrove CL, Wilkin TA, Knowles SC, Day KP, Sheldon BC. 2007. Within-population variation in prevalence and lineage distribution of avian malaria in blue tits, *Cyanistes caeruleus*. Mol Ecol. 16:3263–3273.

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Todos llevamos nuestra posible perdición pegada a los talones

Rosa Montero

El comportamiento de escape es llevado a cabo por las aves capturadas por un depredador, así, una mayor intensidad de este tipo de comportamiento podría incrementar las probabilidades de escapar de los depredadores. Es sabido que, para aumentar su eficacia biológica, los parásitos provocan efectos negativos en el hospedador. Parásitos transmitidos por vectores, como la malaria, obtendrían una mayor probabilidad de transmisión mediante la manipulación del comportamiento de sus hospedadores. Varios estudios han mostrado que el parásito de la malaria puede manipular a los vectores provocando así un aumento en su transmisión. Sin embargo, son pocos los estudios que analizan el posible cambio que el parásito de la malaria podría provocar en el comportamiento del hospedador, incrementándose así la dispersión del parásito. En este estudio analizamos el comportamiento de las aves en la naturaleza y en cautividad con el objetivo de determinar si *Plasmodium relictum* puede manipular el comportamiento de escape en uno de sus hospedadores más frecuente, el gorrión común *Passer domesticus*. Primero analizamos la relación entre el parásito de la malaria y el comportamiento de escape de gorriones en libertad, no encontrándose diferencias en el peso o en el comportamiento de escape entre gorriones infectados y no infectados. En segundo lugar, determinamos experimentalmente si el parásito de la malaria puede manipular el comportamiento de escape de los gorriones, encontrándose que la intensidad de picar y la inmovilidad tónica descendieron en los gorriones a los que se les eliminó la carga parásita mediante drogas anti-maláricas. Estos resultados sugieren que gorriones infectados muestran una mayor intensidad de comportamiento de escape, lo cual podría incrementar sus probabilidades de escapar de depredadores y, además, beneficiaría al parásito ya que se incrementarían sus oportunidades de transmisión.

Palabras clave: picar, gorrión común, *Plasmodium relictum*, depredadores, inmovilidad tónica.

Do malaria parasites manipulate the escape behaviour of their avian hosts? An experimental study

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ABSTRACT

Escape behaviour is the behaviour that birds and other animals display when already caught by a predator. An individual exhibiting higher intensity of such anti-predator behaviour could have greater probabilities of escape from predators. Parasites are known to affect different aspects of host behaviour to increase their own fitness. Vector-transmitted parasites such as malaria parasites should gain by manipulating their hosts to enhance the probability of transmission. Several studies have shown that malaria parasites can manipulate their vectors leading to increased transmission success. However, little is known about whether malaria parasites can manipulate escape behaviour of their avian hosts thereby increasing the spread of the parasite. Here we used both observational and experimental approaches to explore if *Plasmodium relictum* can manipulate the escape behaviour of one of its most common avian hosts, the house sparrow *Passer domesticus*. First, in an observational study we explored the relationship between malaria parasites and escape behaviour in wild sparrows. We found no significant difference in either body mass or escape behaviour between infected and un-infected sparrows. Second, we experimentally tested whether malaria parasites manipulate the escape behaviour of their avian host. We showed a decrease in the intensity of biting and tonic immobility after removal of infection with anti-malaria medication compared to pre-experimental behavior. These outcomes suggest that infected sparrows performed more intense escape behaviour, which would increase the likelihood of individuals escaping from predators, but also benefit the parasite by increasing its transmission opportunities.

Key words: biting; house sparrows; *Plasmodium relictum*; predators; tonic immobility

INTRODUCTION

In any animal population, there are behavioural differences among individuals, where some individuals are bolder, more aggressive or more sociable than others. Although individuals may change their aggressiveness or boldness depending on the ecological situation (e.g., predation risk or parasitism), the behaviour of certain individuals is consistent over time and across situations (Gosling, 2001; Réale *et al.* 2010; Sih *et al.* 2004). Predation is a major selection pressure that determines the form and the behaviour of animals (Endler, 1991; Lima, 1998). Therefore, any animal whose behaviour facilitates avoidance of encounters with predators or survival of attacks will increase its fitness (Lind and Cresswell, 2005). In birds, escape behaviour is displayed when an individual is already caught by a predator (Møller *et al.* 2011). An individual exhibiting such anti-predator behaviour increases the probability of being released by biting, struggling, losing feathers, emitting alarm or distress calls or displaying tonic immobility (Högstedt, 1983; Edelaar *et al.* 2012). Hence, individuals with higher intensity of escape behaviour may have enhanced probabilities of escape from predators (Møller *et al.* 2011).

Parasites exert intense selection on their avian hosts (Loye and Zuk, 1991). Therefore, natural selection is expected to favour parasites with mechanisms that enhance their transmission success. Parasites can affect aspects of host behaviour in ways that increase their own fitness (Moore, 2002; Schmid-Hempel, 2011). The behavioural manipulation hypothesis posits that manipulation of host behaviour by parasites confer fitness benefits to the parasite, usually by increasing transmission success compared to conspecifics that are unable to modify host behaviour (Lefèvre *et al.* 2008; Poulin, 2010).

Avian malaria and related haemosporidians are abundant and diverse parasites infecting several hundred species of birds in almost all continents. *Plasmodium* species are among the most pathogenic species of avian malaria, being responsible for mass mortality, population declines and even extinctions of many bird species (Van Riper III *et al.* 1986; Valkiūnas, 2005). These parasites are transmitted from infected to uninfected hosts by blood-sucking arthropods. Their life cycles are complex, involving sexual stages in the vector and asexual stages in the vertebrate host. Theory predicts that parasites that are dispersed by vectors should gain by manipulating their vector to their own advantage (Schmid-Hempel, 2011). Thus *Plasmodium gallinaceum* is able to increase the biting rate of its vector, the mosquito *Aedes aegypti* leading to an increase in its transmission success (Koella *et al.* 2002). Moreover, an increase in the attractiveness of infected hosts to the vector should also enhance the probability of transmission from the vertebrate host to the arthropod vector (Hamilton and Hurd, 2002). Following this idea, Cornet *et al.* (2013a) experimentally demonstrated that infected birds attract significantly more vectors than uninfected ones, suggesting that malaria parasites manipulate the behaviour of vectors to increase their own transmission. Furthermore, alterations in the behaviour of vertebrate hosts may also benefit

parasite transmission. For example, experimental mice carrying *Plasmodium* gametocytes in their blood showed the weakest mosquito-repellent behaviour, thus allowing mosquitoes to ingest infective forms of the malaria parasites and contributing to transmission of the malaria parasites (Day and Edman, 1983).

However, little is known about whether malaria parasites can manipulate escape behaviour of their avian hosts with the aim of promoting the spread of the parasite. So far, only a recent study has explored the association between haemosporidian infection and escape behaviour of avian hosts. Garcia-Longoria *et al.* (2014) showed a positive relationship between the prevalence of *Leucocytozoon* and *Haemoproteus* and the intensity of host escape behaviour in 89 species of birds, but did not detect any correlation between *Plasmodium* and such anti-predator behaviour. Here we explore if *Plasmodium relictum*, a widespread and highly pathogenic haemosporidian parasite, can manipulate the escape behaviour of one of its most common avian hosts, the house sparrow *Passer domesticus* (Marzal *et al.* 2011). With this aim we conducted observational and experimental studies of this bird-malaria system. First, we explored the relationship between prevalence and intensity of infection by malaria parasites and escape behaviour in wild sparrows. If the effects of malaria infection on host manipulation are perceptible under natural conditions, then we should expect a positive relationship between *Plasmodium* infection and the intensity of escape behaviour, presumably to preserve future transmission opportunities. Second, we experimentally tested whether malaria parasites can manipulate the escape behaviour of their avian host by breaking up any correlation with potentially confounding variables. If *P. relictum* is able to manipulate anti-predator behaviour in order to increase its probability of transmission, then we should predict a decrease in the intensity of escape behaviour after removal of the infection with anti-malaria medication.

MATERIALS AND METHODS

Study site and sample collection

The study was carried out in a population of house sparrows in the university campus of Badajoz (38°52'N, 6°58'W), southwest Spain during November-December 2012. We captured 101 adult house sparrows with mist-nets and recorded their body mass with a Pesola spring balance to the nearest 0.1 g. All birds were individually identified with numbered metal rings. One microcapillary of blood (70 μl) was obtained from the brachial vein of each individual and stored in 500 μl of SET buffer (0.15 M NaCl, 0.05 Tris, 0.001 M EDTA, pH 8.0) until DNA extraction. We also obtained a blood smear from each individual in order to estimate parasite intensity (the number of parasites per individual host). Seven behavioural variables (see below for detailed description) were assessed before the bird was bled and released in the aviaries of the Experimental Garden in the University of Extremadura. After initial molecular screening of haemosporidian infection, we selected 32 haemosporidian-infected sparrows and 38 non-infected individuals for experiments. The remaining non-infected sparrows (N = 31) were released no later than three days after capture.

Anti-malaria treatment

House sparrows were placed in the aviary where each cage (3.5 x 1.5 x 2.5 m) contained a maximum of eight individuals. Birds were provided with water and food *ad libitum* and they stayed two weeks in the aviaries in order to achieve acclimatization. Due to a possible influence of drugs on the behaviour of sparrows, individuals were randomly assigned to one of two treatments independently of infection status: (1) a control group of 33 individuals (14 infected + 19 non-infected sparrows) that were injected with 0.2 ml phosphate buffered saline (PBS); or an experimental group of 37 individuals (18 infected + 19 non-infected sparrows) that were sub-cutaneously injected with 0.02 mg of Primaquine + 1.4 mg of Chloroquine diluted in 0.2 ml of saline solution (Remple, 2004). We also provide a Malarone™ treatment with fixed – dose combination of 250 mg of atovaquone and 100 mg of proguanil hydrochloride to individuals of the treatment group (Palinauskas *et al.* 2009). One dose contained 0.24 mg of Malarone™ dissolved in 50 µl of drinking water in the water dispensers. We provided the same quantity of water in the dispensers of the control group, but without Malarone. Four infected birds died two weeks after inoculation, so the sample size of the control and treatment group was finally reduced to 31 (12 infected + 19 non-infected sparrows) and 35 (16 infected + 19 non-infected sparrows) individuals, respectively. Immediately before treatment, we recorded body mass of all individuals and took a second blood sample for haemosporidian analysis.

Finally, three weeks after inoculation we took a third blood sample to verify the effectiveness of the anti-malaria treatment. In addition, body mass and the seven behavioural variables were recorded again to assess for an effect of haemosporidian infection on body mass and escape behaviour of individuals.

Intensity of blood parasites

Blood samples were fixed in absolute methanol and stained with Giemsa. The intensity of *Plasmodium* parasites was quantified as the number of parasites per 10,000 erythrocytes under 1,000· magnification with oil immersion (Godfrey *et al.* 1987).

Molecular detection of blood parasite infections

Haemosporidian parasites (*Plasmodium* spp. and *Haemoproteus* spp.) were detected from blood samples using molecular methods (Bensch *et al.* 2000; Waldenström *et al.* 2004). DNA from the avian blood samples were extracted in the lab using the standard phenol / chloroform / isoamylalcohol method (Sambrook *et al.* 2002). Diluted genomic DNA (25 ng/µl) was used as a template in a polymerase chain reaction (PCR) assay for detection of the parasites using nested PCR-protocols described by Waldenström *et al.* (2004). The amplification was evaluated by running 2.5 µl of the final PCR on a 2% agarose gel. All PCR experiments contained one negative control for every eight samples. In the very few cases of negative controls showing signs of amplification (never more than faint bands in agarose gels), the whole PCR-batch was run again to make sure that all positives were true. Positive amplifications were sequenced in order to select the individuals infected by *Plasmodium relictum*.

Behavioural variables

Seven aspects of escape behaviour were assessed before a bird was bled. All these variables were recorded in the aviary before and after treatment. Several of these variables have been associated with susceptibility to predation by hawks and cats (Møller *et al.* 2011). It is important to emphasise that the different components of escape behaviour are mostly independent of each other (Møller *et al.* 2011). The seven behavioural variables were defined as follows:

(1) Wriggle score. We scored how much the bird struggled while being held in a hand (a score of 0—no movement, 1—moves rarely, 2—moves regularly, but not always, 3—moves continuously). Individuals that wriggle more may more readily escape from a predator compared to individuals that stay calm.

(2) Biting. Whether the bird did not bite, when we held our right hand index finger in front of the beak, we gave a score of 0, and if it did a score of 1. We presume that a higher frequency of biting entails an elevated probability of escape from a predator because the predator loses its grip when re-directing its attention towards the biting prey.

(3) Feather loss. While the bird was handled, if it lost feathers we gave a score of 1, or 0 if it did not. Feather loss may result in predators losing their grip of a prey (Møller *et al.* 2006).

(4) Fear scream. While the bird was handled, if it gave a fear scream (a score of 1) or not (a score of 0). Birds giving fear screams attract the attention of secondary predators thereby increasing the probability of escape once captured (Högstedt, 1983; Møller and Nielsen, 2010).

(5) Tonic immobility. At the end of the above procedure we placed the bird, just before it was released, with our right hand on its back on our flat left hand. When the bird was lying still, we removed the right hand and recorded the time until the bird righted itself and flew away. We allowed tonic immobility up to 30 s, and if the bird had not left yet, we terminated the trial. Tonic immobility is a standard measure of fear in poultry research with both environmental and genetic components (Hoagland, 1928; Jones, 1986; Boissy, 1995; Forkman *et al.* 2007). Recently, Edelaar *et al.* (2012) showed that tonic immobility is related to personality and anti-predation behaviour because it is a measure of boldness toward predators. The longer time a bird stays, the higher its level of fear. Tonic immobility has a strongly bimodal distribution, with most individuals having tonic immobility of 0–5 s, but some 10–20% having 25–30 s (Møller *et al.* 2011).

(6) Alarm call. When the bird departed from our hand whether it gave an alarm call (a score of 1) or not (a score of 0). It has been suggested that the function of this call is to distract the predator or to warn conspecifics (Charnov and Krebs, 1975; Platzen and Magrath, 2004).

(7) Breath rate. Carere and van Oers (2004) showed that breath rate is a signal of acute stress in birds when handled. We recorded the number of inhalations during 30 seconds while the bird was held in the hand. According to

Carere *et al.* (2001) we should expect a higher number of inhalations with a higher level of fear.

Statistical procedures

We calculated the repeatability of the behavioural variables in order to confirm the reliability of our measurements. We used the program R-2.15 (R Development Core Team, 2011) with the rptR package to calculated the repeatability of binary variables by using a GLMM with logit-link and multiplicative overdispersion (Nakagawa and Schielzeth, 2010). We used an ANOVA to determine differences in quantitative variables (body mass) and chi-square tests to determine differences in qualitative variables (wriggle score, biting, tonic immobility, feather loss and alarm call) between infected and non-infected individuals. Previous studies have criticized the use of Bonferroni correction of multiple statistical tests (Moran, 2003; Nakagawa, 2004; Garamszegi, 2006; Garamszegi *et al.* 2009) because it could increase the risk of committing Type II errors (Nakagawa and Cuthill, 2007). It has been suggested that effect sizes and confidence intervals (CIs) reliably reveal the biological importance of results (Nakagawa, 2004). Hence, we applied the effect size approach in the analyses of the observational ata in order to disclose the magnitude of our results. To calculate effect sizes, we used the corresponding df to calculate *r* and standard errors (based on *r*) to approximate the corresponding CIs (Nakagawa and Cuthill, 2007). 95% CIs are presented as lower/upper limits. We also present significance levels for illustrative purposes. In behavioural studies, the following benchmark is used for interpretations: $r \approx 0.1$ is a small effect, $r \approx 0.3$ a intermediate effect and $r \approx 1.5$ a strong effect (Cohen, 1988; Møller and Jennions, 2002). The magnitude of effects in biological studies is typically intermediate accounting for 5-7% of the variance, thus being equivalent to effect sizes of 0.22-0.26 (Møller and Jennions, 2002).

With the aim to test the hypothesis that malaria infection could modify the escape behaviour of house sparrows, we analysed the variation in escape behaviour before and after the administration of anti-malaria medication or placebo in malaria infected sparrows (N = 56 observations taken from 28 individuals). We also tested this hypothesis separately in non-infected sparrows that received anti-malaria medication or placebo (N = 76 observations from 38 individuals). Therefore, the second group was used as a second control to ensure that the application of the medication was not the cause of possible changes in escape behaviour. We used a linear mixed model approach to analyses these two subsets of data. The dependent variables were the behavioural variables modelled with the appropriate error structure and link functions (i.e., binomial errors for biting, feather loss, alarm call and distress and Poisson errors for wriggle and tonic immobility). The input variables introduced in the models were body mass, tarsus length, sex, time (included as a factor with two levels, i.e., before-after the application of the treatment), treatment (PBS-medication) and the interaction term between time and treatment. Body mass and tarsus length were included to control for difference among individuals in body condition

that could influence escape behaviour and therefore they were treated as confounding variables. We predicted that if the treatment have an effect on behaviour then the interaction term between treatment and time should be statistical significant. The significant of this interaction term were tested comparing with a Likelihood Ratio Test (LRT) the global model with a reduced model on which the interaction term were previously removed leaving all other input variables in the reduced model. In these models we also included the aviary and subject identification as random terms to control for these source of variation and to control for pseudo-replication caused because we have two observations of escape behaviour (i.e., before and after the application of the treatment) taken from the same individual. The models concerning the dependent variables "alarm call" and "feather loss" did not converge because the data was unbalanced with very few experimental or control individuals giving an alarm call or losing a feather. Therefore, we excluded these escape behavioural variables in the analyses concerning our experimental approach.

ETHICAL NOTE

All the experiments comply with the current laws of Spain, where the experiments were performed.

RESULTS

Repeatability of behavioural variables

Repeatability based on multiple measures for each behavioural variable within individuals is reported in Table 1. Six out of seven measures of escape behaviour of individuals at repeated captures were consistent (repeatabilities ranged from 0.53 to 0.93). Only breath rate did not have a high repeatability. Hence, we excluded breath rate from further analyses in order to avoid biased results.

Relationship between malaria parasites and escape behaviour under natural conditions

We analyzed 101 blood samples from house sparrows searching for haemosporidian parasites. We found 69 (68.3 %) non-infected individuals and 32 (31.7 %) individuals infected with blood parasites. Mean *Plasmodium* parasitemia was 13.9 infected erythrocytes per 10,000 scanned erythrocytes (0.139%). There was a non-significant difference in body mass between infected and non-infected individuals ($F_{1,\ 100}$ = 0.90, P = 0.34, r = 0.096, 95% CI = -0.101/0.287). Likewise, effect size between infected and non-infected house sparrows in escape behaviour was small accompanied by a rather wide range of CIs (Table 2).

Table 1 . Summary statistics for escape behaviour within individual house sparrows and repeatability (*R*). SE indicates standard error.

Variable	Mean (SE)	Range	*R*	95% CI
Wriggle	0.177	0-3	0.530	0.020, 0.573
Biting	0.240	0-1	0.655	0.003, 0.868
Feather loss	0.156	0-1	0.882	0.479, 0.977
Tonic immobility (s)	0.299	0-30	0.931	0.040, 0.936
Distress call	0.290	0-1	0.896	0.001, 0.905
Alarm call	0.225	0-1	0.893	0.213, 0.971
Breath rate	0.193	0-30	-0.21	-0.609, 0.188

Table 2 . Effect size between infected and non-infected house sparrows in escape behaviour under natural conditions. Sample size was 101 house sparrows.

				95% CI		
Variable	**χ^2**	**df**	**Effect size**	**Lower**	**Upper**	***P***
Wriggle score	1.85	21	0.053	-0.144	0.246	0.60
Distress call	0.89	7	0.013	-0.182	0.208	0.37
Biting	0.02	7	0.015	-0.180	0.210	0.88
Feather loss	0.91	7	0.097	-0.099	0.287	0.33
Tonic immobility (s)	1.31	7	0.115	-0.081	0.304	0.25
Alarm call	0.80	7	0.090	-0.107	0.280	0.37

Relationship between malaria parasites and escape behaviour under experimental conditions

Escape behaviour under experimental conditions

Effects of the medication in prevalence and intensity of blood parasites

As expected from the anti-malarial treatment, there was a decrease in prevalence of *Plasmodium* infection in medicated sparrows with an intermediate effect (McNemar N = 16; χ^2_{15} = 14.06, P < 0.001, r = 0.741, 95% CI = 0.389/0.904), while for controls there was no change in prevalence of infection (McNemar N = 12; χ^2_{11} = 0.001, P = 1.00, r = 0.000, 95% CI = -0.442/0.442). Likewise, parasite load decreased in infected individuals from the experimental group (change in parasite load (SE) = 12.11 (22.78) Wilcoxon matched-pairs signed-ranks test: N = 16; z = -3.73, P = 0.000, r = 0.678 , 95% CI = 0.337/0.862), while it remained similar in infected individuals from the control group (change in parasite load (SE) = 15.00 (28.20) Wilcoxon matched-pairs signed-ranks test: N = 12; z = -1.28, P = 0.201, r = 0.231 , 95% CI = -0.165/0.654).

Effects of the malaria infection in escape behaviour

We found that two out of the seven variables related to escape behaviour changed in infected house sparrows treated with anti-malaria medication. On the contrary, the escape behaviour of non-infected house sparrows did not change after the medication. Specifically, we found that the probability of biting significantly decreased in malaria infected birds after the experimental reduction of the prevalence and intensity of *Plasmodium relictum* (Fig. 1). This was supported because the interaction term between time and treatment was statistically significant (LRT = 4.59, df = 1, P = 0.032) (Table 3). Likewise, we found that the tonic immobility significantly increased in infected sparrows that received an anti-malaria treatment (Fig. 2). This was supported because the interaction term between time and treatment was statistically significant (LRT = 47.7, df = 1, P < 0.001) (Table 3). These results were not confounded by either the body condition of the birds before the treatment or the aviaries where the experiment was performed, because we controlled for this source of variation in our mixed model approach (Table 3).

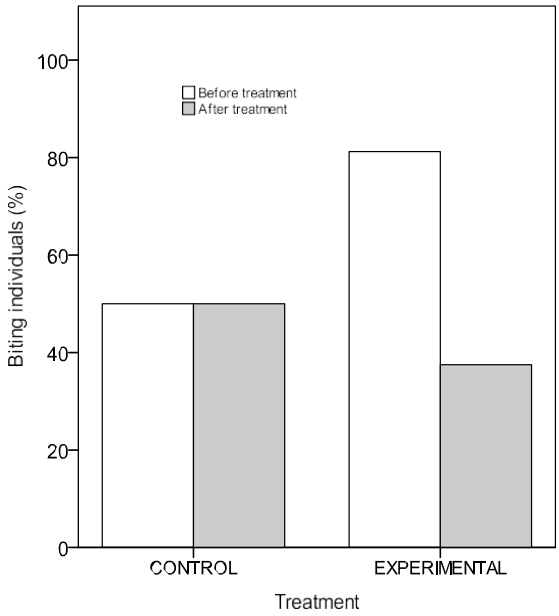

Figure. 1 Percentage of biting individual house sparrows (%) in control and experimental groups before and after anti-malaria treatment. Sample sizes are 20 and 16 sparrows, respectively.

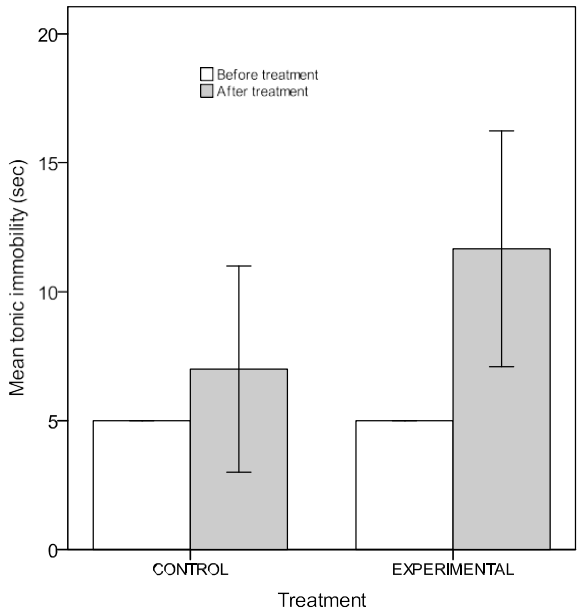

Figure. 2 Mean tonic immobility (sec) ± SD in control and experimental groups of house sparrows before and after anti-malaria treatment. Sample sizes are 20 and 16 sparrows, respectively.

On the contrary, we found no effect of the anti-malaria medication *per se* on the escape behaviour of sparrows. In this sense, any of the variables related to escape behaviour significantly varied among the non-infected sparrows after the administration of anti-malaria medication or PBS (all $P > 0.1$) (Table 4). These results were not confounded by either the body condition of the birds before the treatment or the aviaries where the experiment was performed, because we controlled for this source of variation in our mixed model approach (Table 4).

DISCUSSION

The behavioural manipulation hypothesis posits that parasites can change the behaviour of their hosts to their own selective advantage, usually by increasing their reproductive fitness (Thomas *et al.* 2005; Lefèvre *et al.* 2008). Here we have tested this hypothesis using both observational and experimental approaches in a bird host–malaria system. While the observational approach did not show any difference in escape behaviour between infected and un-infected house sparrows, the experiment clearly showed that biting and tonic immobility changed after clearance of the infection. Moreover, we showed that the anti-malaria drug by itself did not provoke a change in behaviour.

Individual measurements of escape behaviour in birds when captured by a human were consistent across repeated measurements in a population of wild-caught house sparrows. The repeatability of escape behaviour was high for behavioural traits, which confirms the validity of our field estimates, as did previous studies of repeatability of animal behaviour (Dingemanse, 2002; Biro, 2012).

Ecological factors regulating variation in behaviour have been the focus of recent studies (e.g. Réale *et al.* 2010; Dunn *et al.* 2011). It has been postulated that parasites could be one such ecological factor modulating and causing changes in host behaviour (Barber and Dingemanse, 2010; Boyer *et al.* 2010). Several studies have shown that a wide range of protozoan and metazoan parasites induces changes in the behaviour of their hosts (Moore, 2002; Tomás *et al.* 2007; Poulin, 2010, 2013). However, some studies failed to show a

Table 3. Effects of anti-malaria treatment on escape behaviour of infected House sparrows. Sample size was 28 individuals.

Variable	Estimate	SE	*z* value	*P*
Biting				
Sex (Male)	-0.361	0.666	-0.54	0.58
Body Mass	0.229	0.193	1.18	0.23
Tarsus	0.083	0.408	0.20	0.83
Time (before)	-0.159	0.892	-0.17	0.85
Treatment (PRQ)	0.815	0.863	0.94	0.34
Time*Treatment	-2.679	1.245	4.59	0.032*
Distress				
Sex (Male)	0.781	0.827	0.944	0.34
Body Mass	-0.065	0.237	-0.277	0.78
Tarsus	-0.424	0.503	-0.843	0.39
Time (before)	-0.957	1.046	-0.915	0.36
Treatment (PRQ)	-1.586	1.015	-1.561	0.11
Time*Treatment	0.220	1.653	0.017	0.894
Tonic immobility				
Sex (Male)	-0.186	0.444	-0.419	0.67
Body Mass	0.169	0.082	2.056	0.039*
Tarsus	-0.327	0.273	-1.197	0.231
Time (before)	-0.460	0.178	-2.578	0.009
Treatment (PRQ)	-0.733	0.472	-1.552	0.120
Time*Treatment	1.579	0.234	47.77	<0. 0001***
Wriggle				
Sex (Male)	0.072	0.208	-0.150	0.72
Body Mass	-0.054	0.062	-0.874	0.38
Tarsus	0.118	0.127	0.934	0.35
Time (before)	0.271	0.310	0.876	0.38
Treatment (PRQ)	-0.154	0.304	-0.508	0.61
Time*Treatment	0.052	0.411	0.016	0.89

Table 4. Effects of anti-malaria treatment on escape behaviour of non-infected House sparrows. Sample Size was 38 individuals.

Variable	Estimate	SE	*z* value	*P*
Biting				
Sex (Male)	0.776	0.996	0.779	0.43
Body Mass	-0.226	0.245	-0.921	0.35
Tarsus	0.204	0.472	0.433	0.66
Time (before)	-0.145	0.929	-1.232	0.21
Treatment (PRQ)	0.226	1.158	0.196	0.84
Time*Treatment	0.374	1.178	0.08	0.77
Distress				
Sex (Male)	-0.733	4.876	-0.150	0.88
Body Mass	0.604	0.915	0.660	0.50
Tarsus	-0.986	2.103	-0.469	0.63
Time (before)	-0.472	2.175	-0.217	0.82
Treatment (PRQ)	-0.057	4.660	-0.012	099
Time*Treatment	-0.368	2.404	0.000	1.00
Tonic immobility				
Sex (Male)	0.369	0.599	0.616	0.53
Body Mass	-0.067	0.050	-1.340	0.18
Tarsus	0.029	0.255	0.116	0.90
Time (before)	-0.145	0.152	-0.954	0.34
Treatment (PRQ)	0.446	0.597	0.747	0.45
Time*Treatment	0.187	0.159	1.390	0.23
Wriggle				
Sex (Male)	0.065	0.178	0.366	0.71
Body Mass	0.023	0.049	0.469	0.63
Tarsus	-0.024	0.089	-0.268	0.78
Time (before)	-0.013	0.233	-0.056	0.95
Treatment (PRQ)	-0.151	0.244	-0.618	0.53
Time*Treatment	0.026	0.327	0.006	0.90

relationship between infection status and behaviour of hosts (Poulin, 2010; Cornet *et al.* 2013b; Garcia-Longoria *et al.* 2014). Likewise, in our observational study we found no differences in escape behaviour between infected and un-infected sparrows. It has been proposed that changes in behaviour may be positively correlated with parasite load, where higher intensities of infection should induce greater alterations in behaviour (Thomas and Poulin, 1998; Shirakashi and Goater, 2002). Because *Plasmodium* load varies during infection, it is possible that the dynamics of infection could be the cause of the lack of differences in escape behaviour between infected and non-infected birds in the wild population of house sparrows. Avian malaria parasites of the genus *Plasmodium* cause substantial morbidity and mortality in natural populations during the brief acute stage of a haemosporidian infection (Valkiūnas, 2005), when parasites usually appear in the blood at high density (Atkinson and Van Riper III, 1991; Valkiūnas, 2005). However, it is common that long-term chronic infections developed in those individuals that survive the acute stage. In these individuals parasites persist at low densities in blood stream and are thought to be controlled by acquired immunity (Atkinson and Van Riper III, 1991; Zehtindjiev *et al.* 2008). Alternatively, in temperate regions, the lowest indices of infection intensity of haemosporidians are observed in birds in winter because suitable vectors for parasite transmission are absent (Valkiūnas, 2005). These outcomes are in accordance with our results where naturally infected birds showed very low parasitemia. This small intensity of infection could explain why we failed to show effects of *Plasmodium* parasites in wild sparrows during winter.

Avian malaria parasites were considered for many years to have low pathogenicity because many studies failed to show a correlation between haemosporidian infections and fitness components of their hosts (Fallis and Desser, 1997; Dufva and Allander, 1995; Dawson and Bortolotti, 2000). However, the demonstration of effects of parasites requires an experimental approach (Keymer and Read, 1991; Marzal *et al.* 2005; Knowles *et al.* 2010). Interestingly, we found that two components of anti-predator escape behaviour changed with the reduction in malaria infection. Specifically, medicated sparrows showed a lower frequency of biting behaviour and spent more time in tonic immobility before flying away. We also found that body mass of infected birds increased after treated them with an anti-malaria drug. However, non-infected individuals also increased their body mass, independently to the treatment. Therefore, the change in behaviour we found was independent of change in body mass since also non-infected individuals change their body mass during the course of the study. Additionally, we did not find any change in behaviour in non-infected individuals treated either with anti-malaria medication or PBS, as expected. The direction of the change in behaviour in infected individuals treated with anti-malaria medication shows a trend towards parasite-free individuals being more scared or shy. Numerous studies have shown that parasites can modify specific anti-predator behaviours to increase their own fitness (see reviews in Moore, 2002; Lafferty and Shaw, 2013). These modifications include examples in

which parasites alter the behaviour of their intermediate hosts in ways that favour predation of infected hosts, thus enhancing trophic transmission. For example, a rodent infected with *Toxoplasma gondii* is known to lose fear against predators (definitive host), thus increasing the transmission of the parasite to its final host (Berdoy *et al.* 2000; Vyas *et al.* 2007; Webster and McConkey, 2010). In the case of malaria parasites, the death of an infected host by a predator entails the end of transmission of the parasite. Here, we showed that sparrows were more aggressive against the predator and performed more intense escape behaviour when infected with malaria parasites. Theoretically, these changes in host behaviour should benefit the parasite. In this sense, intense escape behaviour that allows a bird to escape from a predator attack would increase the likelihood of survival (Møller *et al.* 2011). Indirectly, this would also benefit the parasite by increasing their transmission opportunities, the key factor determining parasite fitness. To the best of our knowledge, this is the first study experimentally showing a modification in host escape behaviour provoked by a malaria parasite.

The manipulation behaviour hypothesis posits that parasites may induce behavioural changes in their host in ways to benefit their transmission to other hosts and hence increase their own fitness (Lefèvre *et al.* 2008; Poulin, 2010). But hosts usually do not obtain any benefit from these parasite manipulations (Levri, 1995; Vyas *et al.* 2007). However, our findings revealed that sparrows could also benefit from *Plasmodium* manipulation. In this sense, we showed that malaria infected sparrows performed more intense escape behaviour, which would indeed increase their likelihood of escape from predators and survival. If the performance of intense escape behaviour may clearly benefit an individual host, the question raised from here is why sparrows do not always exhibit a maximum in this behaviour, regardless to their infection status. One explanation could be that this anti-predatory behaviour should have some associated costs, and hence there could be an optimization of benefits associated with escape weighed against costs of the performance of the behaviour. In this sense, it has been shown that aggressive behaviour in birds can incur short-term costs in terms of energy and risk of injuries (Brown, 1997; Viera *et al.* 2011), which may result in long-term fitness costs (Hagelin, 2002). Alternatively, escape behaviour may show behavioural plasticity, where the expression of some behavioural traits (e.g. shyness and boldness) may vary within and among individuals across different environmental conditions (Réale, 2007; Stamps and Groothuis, 2010; Poulin, 2013). Future studies examining the costs and benefits of escape behaviour in parasitized birds are desirable for understanding these variations.

Another remaining question concerns the identification of the mechanisms that *Plasmodium* may use to enhance the escape behaviour of house sparrows. Although the molecular mechanisms are still poorly understood, three main mechanisms have been proposed to be used by parasites to alter host behaviour following infection (Adamo and Webster, 2013; Poulin, 2010). First, some parasites secrete substances capable of altering neuronal activity of the host provoking a change in host

behaviour. For instance, *Schistosoma mansoni*, a trematode, secretes opioid peptides altering neuronal functions in its host (Kavaliers *et al.* 1999). Second, parasites can also modify the behaviour by interfering with physiological and biochemical pathways, inducing an indirect change in the behaviour of the infected host. For example, larval stages of helminths can promote changes in concentrations of serotonin, dopamine and others neurotransmitters in the brain of hosts, thereby modifying their behaviour (Poulin *et al.* 2003). Third, parasites may use genomics and proteomics to induce changes in the brain of the host. For example, *T. gondii*, a obligate parasitic protozoan, secretes a protein kinase in cells of an infected host thereby altering the expression of genes involved in immune function and neural signalling of the host (Hakimi and Cannella, 2011). We do not know the mechanism used by *Plasmodium* to modify the behaviour of its vertebrate hosts. *Plasmodium* parasites are known to alter the behaviour of their intermediate hosts. For example, it has been experimentally shown that *Plasmodium gallinaceum* affects the host-seeking behaviour of its mosquito vector *Aedes aegypti* to increase its transmission success (Koella *et al.* 2002). More recently, malaria infected mosquitoes have been shown to express enhanced attraction to human odour (Smallegange *et al.* 2013). Such modifications could be produced by alteration of an enzyme in the salivary glands of mosquitoes that hamper the blood meal process (Rossignol *et al.* 1984) and alterations in proteins in the head of sporozoite-infected mosquitoes indicating a possible dysfunction in the central neural system (Lefèvre *et al.* 2007).

In our correlational study we found no differences in escape behaviour between infected and un-infected sparrows while in the experimental study we found that behaviour changed after experimental elimination of malaria parasites. However, such differences are commonplace. The most likely reason for the apparent discrepancy is that one or more confounding variables obscured any relationships in the correlational study. Thus, the effects of these confounding variables were eliminated by the experiment. In conclusion, this is the first study to experimentally show a modification in host escape behaviour provoked by a malaria parasite, as expected for parasite manipulation of host behaviour. Further studies of mechanisms involved in parasite-induced changes in escape behaviour may provide powerful tools for understanding bird–malaria interactions.

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REFERENCES

Adamo, S. A. and Webster, J. P. (2013). Neural parasitology: how parasites manipulate host behaviour. *The Journal of experimental biology* **216**, 1–2.

Atkinson, C. T. and Van Riper III, C. (1991). Pathogenicity and epizootiology of avian haematozoa: Plasmodium, Leucocytozoan, and Haemoproteus. In *Bird-Parasite Interactions: Ecology, evolution, and behaviour* (ed. Loye, J. E. and Zuk, M.), pp 19-48. Oxford University Press, London, UK.

Barber, I. and Dingemanse, N. J. (2010). Parasitism and the evolutionary ecology of animal personality. *Philosophical transactions of the Royal Society of London* **365**, 4077–88.

Bensch, S., Stjernman, M., Hasselquist, D., Ostman, O., Hansson, B., Westerdahl, H. and Pinheiro, R. T. (2000). Host specificity in avian blood parasites: a study of *Plasmodium* and *Haemoproteus* mitochondrial DNA amplified from birds. *Proceedings. Biological sciences* **267**, 1583–1589.

Berdoy, M., Webster, J. P. and Macdonald, D. W. (2000). Fatal attraction in rats infected with *Toxoplasma gondii*. *Proceedings. Biological sciences* **267**, 1591–1594.

Biro, P. A. (2012). Do rapid assays predict repeatability in labile (behavioural) traits? *Animal Behaviour* **83**, 1295–1300.

Boissy, A. (1995). Fear and fearfulness in animals. *The Quarterly review of biology* **70**, 165–191.

Boyer, N., Réale, D., Marmet, J., Pisanu, B. and Chapuis, J. L. (2010). Personality, space use and tick load in an introduced population of Siberian chipmunks *Tamias sibiricus*. *The Journal of animal ecology* **79**, 538–547.

Brown, C. R. (1997). Purple Martin (*Progne subis*). In *The birds of North America* (ed. Pool, A. and Giil. E.). Philadelphia, and the Am&can Ornithologists Union, Whashintong DC, USA.

Carere, C. and Van Oers, K. (2004). Shy and bold great tits (*Parus major*): body temperature and breath rate in response to handling stress. *Physiology & behavior* **82**, 905–912.

Carere, C., Welink, D., Drent, P. J., Koolhaas, J. M. and Groothuis, T. G. (2001). Effect of social defeat in a territorial bird (*Parus major*) selected for different coping styles. *Physiology & Behavior* **73**, 427–433.

Charnov, L. E. and Krebs, R. J. (1975). The evolution of alarm calls: altruism or manipulation? *The American naturalist* **109**, 107–112.

Cohen, J. (1988). *Statistical power analysis for the behavioral science*. Lawrence Erlbaum Associates, Hillsdale.

Cornet, S., Nicot, A., Rivero, A. and Gandon, S. (2013a). Malaria infection increases bird attractiveness to uninfected mosquitoes. *Ecology Letters* **16**, 323–329.

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

Dawson, R. D. and Bortolotti, G. R. (2000). Reproductive success of American kestrels: the role of prey abundance and weather. *The Condor* **102**, 814–822.

Day, J. F. and Edman, J. D. (1983). Malaria renders mice susceptible to mosquito feeding when gametocytes are most infective. *The Journal of parasitology* **69**, 163–170.

Dingemanse, N. (2002). Repeatability and heritability of exploratory behaviour in great tits from the wild. *Animal Behaviour* **64**, 929–938.

Dufva, R. and Allander, K. (1995). Intraspecific variation in plumage coloration reflects immune response in Great tit (*Parus major*) males. *Functional ecology* **9**, 785–789.

Dunn, J. C., Cole, E. F. and Quinn, J. L. (2011). Personality and parasites: sex-dependent associations between avian malaria infection and multiple behavioural traits. *Behavioral Ecology and Sociobiology* **65**, 1459–1471.

Edelaar, P., Serrano, D., Carrete, M., Blas, J., Potti, J. and Tella, J. L. (2012). Tonic immobility is a measure of boldness toward predators : an application of Bayesian structural equation modeling. *Behavioral Ecology* **23**, 619–626.

Endler, J. A. (1991). Interactions between predators and prey. In *Behavioural ecology an evolutionary approach* (ed. Krebs, J. R. and Davies N. B.), pp 169–196. Blackwell, Oxford, UK.

Fallis, A. and S. Desser. (1997). On species of *Leucocytozoon*, *Haemoproteus* and *Hepatocystis*. *Parasitic Protozoa* **3**, 239–266.

Forkman, B., Boissy, A., Meunier-Salaün, M. C., Canali, E. and Jones, R. B. (2007). A critical review of fear tests used on cattle, pigs, sheep, poultry and horses. *Physiology & Behavior* **92**, 340–374.

Garamszegi, L. Z. (2006). Comparing effect sizes across variables: generalization without the need for Bonferroni correction. *Behavioral Ecology* **17**, 682–687.

Garamszegi, L. Z., Calhim, S., Dochtermann, N., Hegyi, G., Hurd, P. L., Jorgensen, C., Kutsukake, N., Lajeunesse, M. J., Pollard, K. A., Schielzeth, H., Symonds, M. R. E. and Nakagawa, S. (2009). Changing philosophies and tools for statistical inferences in behavioral ecology. *Behavioral Ecology* **20**, 1363–1375.

Garcia-Longoria, L., Garamszegi, L. Z. and Møller, A. P. (2014). Host escape behaviour and blood parasite infections in birds. *Behavior Ecology* **25**, 890–900.

Godfrey, R. D., Fedynich, A. M. and Pence, D. B. (1987). Quantification of hematozoa in blood smears. *Journal of wildlife diseases* **23**, 558–565.

Gosling, S. D. (2001). From mice to men: what can we learn about personality from animal research? *Psychological bulletin* **127**, 45–86.

Hagelin, J. C. (2002). The kinds of traits involved in male-male competition: a comparison of plumage, behaviour, and body size in quail. *Behavioral Ecology* **1**, 32-41.

Hakimi, M. Ali. and Cannell, D. (2011). Apicomplexan parasites and subversion of the host cell microRNA pathway. *Trends in Parasitology* **27**, 481–486.

Hamilton, J. and Hurd, H. (2002). *The Behavioural Ecology of Parasites*. CABI Publi, Wallingford, UK.

Hoagland, H. (1928). On the mechanism of tonic immobility in vertebrates. *The Journal of general physiology* **11**, 715–741.

Högstedt, G. (1983). Adaptation Unto Death : Function of Fear Screams. *American Naturalist* **121**, 562–574.

Jones, R. B. (1986). The tonic immobility reaction of the domestic fowl: a review. *World Poultry Science Journal* **42**, 82–96.

Kavaliers, M., Colwell, D. D., Choleris, E. and Ossenkopp, K. P. (1999). Learning to cope with biting flies: rapid NMDA-mediated acquisition of conditioned analgesia. *Behavioral neuroscience* **113**, 126–135.

Keymer, A. R. and Read, A. F. (1991). Behavioural ecology: the impact of parasitism. In *Parasite-Host Associations, Coexistence or Conflict??* (ed. Toft, C. A., Aeschlimann, A. and Bolis, L.), pp 37-61. Oxford University Press, Oxford.

Knowles, S. C. L., Palinauskas, V. and Sheldon, B. C. (2010). Chronic malaria infections increase family inequalities and reduce parental fitness: experimental evidence from a wild bird population. *Journal of Evolutionary Biology* **23**, 557–569.

Koella, J. C., Rieu, L. and Paul, R. E. L. (2002). Stage-specific manipulation of a mosquito's host-seeking behavior by the malaria parasite

Plasmodium gallinaceum. *Behavioral Ecology* **13**, 816–820.

Lafferty, K. D. and Shaw, J. C. (2013). Comparing mechanisms of host manipulation across host and parasite taxa. *The Journal of experimental biology* **216**, 56–66.

Lefevre, T., Thomas, F., Schwartz, A., Levashina, E., Blandin, S., Brizard, J. P., Le Bourligu, L., Demettre, E., Renaud, F. and Biron, D. (2007). Malaria *Plasmodium* agent induces alteration in the head proteome of their *Anopheles* mosquito host. *Proteomics* **7**, 1908–1915.

Lefèvre, T., Roche, B., Poulin, R., Hurd, H., Renaud, F. and Thomas, F. (2008). Exploiting host compensatory responses: the "must" of manipulation? *Trends in Parasitology* **24**, 435–439.

Levri, E. P. (1995). Parasite-induced change in host behavior of a freshwater snail : parasitic manipulation or byproduct of infection ? *Behavioral Ecology* **10**, 234–241.

Lima, S. (1998). Stress and decision making under the risk of predation: recent developments from behavioral, reproductive, and ecological perspectives. *Advances in the Study of Behavior* **27**, 215–290.

Lind, J. and Cresswell, W. (2005). Determining the fitness consequences of antipredation behavior. *Behavioral Ecology* **16**, 945–956.

Loye, J. E. and Zuk, M. (1991). *Bird-parasite interactions: ecology, evolution and behaviour*. Oxford University Press, Oxford.

Marzal, A., de Lope, F., Navarro, C. and Møller, A. P. (2005). Malarial parasites decrease reproductive success: an experimental study in a passerine bird. *Oecologia* **142**, 541–545.

Marzal, A., Ricklefs, R. E., Valkiūnas, G., Albayrak, T., Arriero, E., Bonneaud, C., Czirják, G. A., Ewen, J., Hellgren, O., Hořáková, D., Iezhova, T. A., Jensen, H., Križanauskienė, A., Lima, M. R., De Lope, F., Magnussen, E., Martin, L. B., Møller, A. P., Palinauskas, V., Pap, P. L., Pérez-Tris, J., Sehgal, R. N. M., Soler, M., Szöllősi, E., Westerdahl, H., Zetindjiev, P. and Bensch, S. (2011). Diversity, loss, and gain of malaria parasites in a globally invasive bird. *PLoS ONE* **6**, 8.

Møller, A. P. and Jennions, M. (2002). How much variance can be explained by ecologists and evolutionary biologists? *Oecologia* **132**, 492–500.

Møller, A. P. and Nielsen, J. T. (2010). Fear screams and adaptation to avoid imminent death: effects of genetic variation and predation. *Ethology Ecology & Evolution* **22**, 183–202.

Møller, A. P., Nielsen, J. T. and Erritzøe, J. (2006). Losing the last feather: feather loss as an antipredator adaptation in birds. *Behavioral Ecology* **17**, 1046–1056.

Møller, A. P., Christiansen, S. and Mousseau, T. (2011). Sexual signals, risk of predation and escape behavior. *Behavior Ecology* **22**, 800–807.

Moore, J. (2002). *Parasites and the behavior of animals*. Oxford University Press, Oxford.

Moran, M. D. (2003). Arguments for rejecting the sequential Bonferroni in ecological studies. *Oikos* **102**, 403–405.

Nakagawa, S. (2004). A farewell to Bonferroni: the problems of low statistical power and publication bias. *Behavioral Ecology* **15**, 1044–1045.

Nakagawa, S. and Cuthill, I. C. (2007). Effect size, confidence interval and statistical significance: a practical guide for biologists. *Biological reviews of the Cambridge Philosophical Society* **82**, 591–605.

Nakagawa, S. and Schielzeth, H. (2010). Repeatability for Gaussian and non-Gaussian data: a practical guide for biologists. *Biological reviews of the Cambridge Philosophical Society* **85**, 935–956.

Palinauskas, V., Valkiūnas, G., Krizanauskiene, A., Bensch, S. and Bolshakov, C. V. (2009). *Plasmodium relictum* (lineage P-SGS1):

Further observation of effects on experimentally infected passeriform birds, with remarks on treatment with Malarone. *Experimental Parasitology* **123**, 134–139.

Platzen, D. and Magrath, R. D. (2004). Parental alarm calls suppress nestling vocalization. Proceedings. *Biological sciences* **271**, 1271–1276.

Poulin, R. (2010). Parasite manipulation of host behavior : an update and frequently asked questions. In *Advances in the Study of Behavior* (ed. Brockmann, J), pp 151-186. Academic Press Burlington, New Jersey.

Poulin, R. (2013). Parasite manipulation of host personality and behavioural syndromes. *The Journal of experimental biology* **216**, 18–26.

Poulin, R., Nichol, K. and Latham, A. D. M. (2003). Host sharing and host manipulation by larval helminths in shore crabs: cooperation or conflict? *International journal for parasitology* **33**, 425–433.

R Development Core Team. (2011). R: A language and environment for statistical computing. R Foundation Statistical Computing.

Réale, D., Dingemanse, N. J., Kazem, A. J. N. and Wright, J. (2010). Evolutionary and ecological approaches to the study of personality. *Philosophical Transactions of the Royal Society of London - Series B: Biological Sciences* **365**, 3937–3946.

Réale, D., Reader, S. M., Sol, D., McDougall, P. T. and Dingemanse, N. J. (2007). Integrating animal temperament within ecology and evolution. *Biological reviews of the Cambridge Philosophical Society* **82**, 291–318.

Remple, D. J. (2004). Intracellular hematozoa of raptors : a review and update and. *Journal of Avian Medicine and Surgery* **18**, 75–88.

Rossignol, P. A., Ribeiro, J. M. and Spielman, A. (1984). Increased intradermal probing time in sporozoite-infected mosquitoes. *The American journal of tropical medicine and hygiene* **33**, 17–20.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (2002). *Molecular cloning: A laboratory manual*. Cold Sprin, New York, USA.

Schmid-Hempel, P. (2011). *Evolutionary parasitology: The integrated study of infections, immunology, ecology and genetics*. Oxford University Press, Oxford, UK.

Shirakashi, S, and Goater, C. P. (2002). Intensity-dependent alteration of minnow (*Pimephales promelas*) behavior by a brain-encysting trematode. *The Journal of parasitology* **88**, 1071–1074.

Sih, A., Bell, A. M., Johnson, J. C. and Ziemba, R. E. (2004). Behavioral syndrome : an integrative overview. *The Quarterly review of biology* **79**, 241–277.

Smallegange, R. C., van Gemert, G. J., van de Vegte-Bolmer, M., Gezan, S., Takken, W., Sauerwein, R. W. and Logan, J. G. (2013). Malaria infected mosquitoes express enhanced attraction to human odor. *PLoS ONE* **8**, 5.

Stamps, J. A. and Groothuis. T. G. G. (2010). Developmental perspectives on personality: implications for ecological and evolutionary studies of individual differences. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences* **365,** 4029–4041.

Thomas, F., and Poulin, R. (1998). Manipulation of a mollusc by a trophically transmitted parasite: convergent evolution or phylogenetic inheritance? *Parasitology* **116**, 431–436.

Thomas, F., F. Renaud, and J. F. Guégan. (2005). *Parasitism and ecosystems*. Oxford Uiversity Press, Oxford, UK.

Tomás, G., S. Merino, J. Moreno, and J. Morales. (2007). Consequences of nest reuse for parasite burden and female health and condition in blue tits, *Cyanistes caeruleus*. *Animal Behaviour* **73**, 805–814.

Valkiūnas, G. (2005). *Avian malaria parasites and other Haemosporidia*. CRC Press, Boca Raton, FL, USA.

Van Riper III, C., Van Riper, S. G., Goff, M. L. and Laird, M. (1986). The epizootiology and ecological significance of malaria in Hawaiian land birds. *Ecological Monographs* **56**, 327–344.

Viera, V. M., Viblanc, V. A., Filippi-Codaccioni, O., Côté, S. D. and Groscolas, R. (2011). Active territory defence at a low energy cost in a colonial seabird. *Animal Behaviour* **82**, 69-76.

Vyas, A., Kim, S. K., Giacomini, N., Boothroyd, J. C. and Sapolsky, R. M. (2007). Behavioral changes induced by *Toxoplasma* infection of rodents are highly specific to aversion of cat odors. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 6442–6447.

Waldenström, J., Bensch, S., Hasselquist, D. and Ostman, O. (2004). A new nested polymerase chain reaction method very efficient in detecting *Plasmodium* and *Haemoproteus* infections from avian blood. *The Journal of parasitology* **90**, 191–194.

Webster, J. P. and McConkey, G. A. (2010). *Toxoplasma gondii*-altered host behaviour: clues as to mechanism of action. *Folia parasitologica* **57**, 95–104.

Zehtindjiev, P., Ilieva, M., Westerdahl, H., Hansson, B., Valkiūnas, G. and Bensch, S. (2008). Dynamics of parasitemia of malaria parasites in a naturally and experimentally infected migratory songbird, the great reed warbler *Acrocephalus arundinaceus*. *Experimental Parasitology* **119**, 99–110.

Invasive avian malaria as an emerging parasitic disease in native birds of Peru

III

Habitat destruction is usually linked to greed and materialism in the developed world

Dian Fossey

Algunas especies de parásitos maláricos que afectan a aves son invasoras y responsables de la pérdida de diversidad aviar a nivel mundial. En este estudio, analizamos la prevalencia y la caracterización genética de la malaria aviar y de parásitos haemosporidios en aves Neotropicales de dos regiones diferentes de Perú. Detectamos una prevalencia del 32.4% en 12 especies de aves. Asimismo, encontramos que el agente patógeno *Plasmodium relictum* SGS1 mostró una distribución muy amplia y además fue el linaje más prevalente detectado en nuestro estudio (39% de las infecciones totales) infectando 8 especies de hospedadores en ambas localidades. Según nuestros datos, el presente estudio es el primero en detectar este patógeno en el continente suramericano. De esta forma, el linaje pSGS1 representaría una posible amenaza para un tercio de todas las especies de aves en el mundo.

Palabras clave: invasión biológica, parásitos sanguíneos, *Haemoproteus*, *Plasmodium*, Suramérica.

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INVASION NOTE

Invasive avian malaria as an emerging parasitic disease in native birds of Peru

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Abstract Some species of avian malaria parasites are invaders and responsible for diversity losses worldwide. Here we analyze the prevalence and genetic characterization of avian malaria and related haemosporidian parasites in Neotropical birds from two different regions of Peru. We detected an overall prevalence of 32.4 % comprising 12 infected bird species. The pathogen Plasmodium relictum SGS1 was widespread and the most prevalent parasite found in our study (39 % of the total infections), infecting 8 host species in both localities. To the best of our knowledge, this is the first report of this invasive pathogen in the mainland Americas, thus representing a possible menace to over one-third of all bird species in the world.

Keywords Biological invasion · Blood parasites · Malaria · Haemoproteus · Plasmodium · South America

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Introduction

Many Emerging Infectious Diseases (EID) are a result of the increasing incidence and impact on host fitness by biological invasions of parasites that have "jumped ship" to novel host species (Hatcher et al. 2012). Malaria parasites (genus Plasmodium) are globally distributed, including several hundred species infecting birds of most species. Plasmodium relictum is among the most pathogenic species of avian malaria, being responsible for mass mortality, population declines and even extinctions of many bird species worldwide after its introduction outside its native range (Van Riper et al. 1986; Valkiūnas 2005). P. relictum lineage pSGS1 is a widespread and actively transmitted parasite lineage in Europe, Africa and Asia (Palinauskas et al. 2007), although recently it has also been recorded infecting native and indigenous birds in Oceania (Howe et al. 2012). P. relictum lineage pSGS1 has been reported in over 60 species of birds, but as of yet, this invasive lineage has not been reported in the mainland Americas (Beadell et al. 2006; Durrant et al. 2006; Merino et al. 2008; Marzal et al. 2011; Lacorte et al. 2013). It is closely related to P. relictum lineage pGRW4 (where lineages are based on partial sequences of the cytochrome b gene), which also has a broad geographical range including New Zealand, Africa, Asia and the Americas (Beadell et al. 2006; Marzal et al. 2011). Both parasites lineages might easily switch to new hosts as they spread into new areas (Beadell et al. 2006; Hellgren et al. 2009).

Fig. 1 Collection sites in Peru

For all these reasons, the International Union for Conservation of Nature (IUCN) classifies avian malaria P. relictum to be among the 100 of the world's worst invasive alien species (Lowe et al. 2000). Hence, the identification of the geographical distribution of P. relictum lineages and their infection prevalences in birds has become essential in order to develop appropriate management strategies to facilitate biodiversity conservation efforts worldwide. Here we analyze the presence of pSGS1 in Neotropical birds from two different areas of Peru; Lima and Huanuco. This system in Peru will allow us to test how the arrival of an introduced Plasmodium species affects birds that have already been exposed to other endemic (native) malaria parasites.

Methods

The study was carried out in two different areas of Peru: Pantanos de Villa wetland Reserve, a RAMSAR protected area in the south of Lima including a

complex of lagoons, pools and marsh areas of Pacific coast (12°12^{0}S, 76°59^{0}W; 10 masl), and Huánuco region, located between the eastern slope of the Andes Mountain Range and the Amazon plain (9°55^{0}S, 76°14^{0}W; 1894 masl) (Fig. 1). In June 2012, 102 individuals from 18 native bird species were caught using mist-nets. Different morphometric characters were measured by the same observer (AM). We captured individuals at each site and recorded their body weight with a Pesola spring balance to the nearest 0.5 g. We measured tarsus length with a digital caliper to the nearest 0.01 mm. We calculated body mass index as the residuals from an ordinary least squares linear regression of body weight against tarsus length (Jakob et al. 1996).

One microcapillary of blood (70 ll) was obtained from the brachial vein of each individual and stored in 500 ll SET buffer (0.15 M NaCl, 0.05 M Tris, 0.001 M EDTA, pH 8.0) until analyses. Blood films were fixed with methanol and stained with Giemsa. Plasmodium and Haemoproteus infections were detected from blood samples using molecular methods (see Hellgren et al. 2004 for detailed description). The obtained sequences of 478 bp of the cyt b were edited, aligned and compared in a sequence identity matrix using the program BioEdit (Hall 1999). P. relictum SGS1 and new lineages were also sequenced from the reverse end using the primer HaemR2.

These molecular analyses were repeated in three independent laboratories to verify the validity and reproducibility of detection of the SGS1 lineage. The obtained results in all the cases were the same. Moreover, we also amplified specific nuclear genes of pSGS1 (García-Longoria, unpublished) to confirm that this finding was not resulting from sporadic lab contamination. The obtained sequences match SGS1 for this fast evolving gene which easily separates SGS1 from other closely related lineages (e.g. GRW4). Up to 40,000 erythrocytes were microscopically examined on each PCR-positive blood films for P. relictum SGS1. Malaria infection was confirmed microscopically in 61.5 % of individuals as early trophozoites and meronts of Plasmodium spp (Fig. 2). Unfortunately, most likely due to low parasitemia, we were not able to identify any gametocytes in the blood films. Blood smears were deposited in the museum collection of Museo de Historia Natural (Universidad Ricardo Palma, Lima, Peru) under the accession numbers PROT-MHN-001–PROT-MHN-007. Intensities of infection were low with parasitemias that did not exceed 1 parasites/10,000 red blood cells. Using the information on the host species and geographical distribution of 1338 parasite lineages provided by MalAvi database (Version 2.0.5 June 2013, Bensch et al. 2009), we studied the host range and geographical distribution of parasites found in our study. We classified as exotic parasite species those lineages that have been previously reported infecting wild birds from other zoogeographical regions (i.e. pSGS1), whereas parasites linages reported in previous studies infecting wild birds from North or South America, but not found in other zoogeographical regions, were considered as native parasite species. New parasite lineages that were found for the first time in the present study were excluded from this categorization. All new DNA sequences have been deposited in GenBank under the accession numbers (KF482344, KF482356, KF482358).

Results and discussion

A total of 102 bird blood samples were examined for haemosporidian infection. We detected an overall prevalence of 32.35 % (33 positive samples) (Table 1). We found 5 Haemoproteus lineages and 5 Plasmodium lineages infecting 12 different bird species (Table 1). Plasmodium relictum SGS1 was widespread and the most prevalent parasite lineage found in our study, infecting 13 individuals from 8 host species in both localities (39.40 % of the total infections) (Table 1). Although the fauna of haemosporidians have been studied irregularly in South America, this parasite lineage may be a recent invader because it has not been previously reported in any of the 39 studies analyzing avian malaria and related haemosporidian parasites from 213 native and introduced bird species from 17 orders covering almost the entire geographical range from North and South America (MalAvi database, version 2.0.5 June 2013, Bensch et al. 2009). Therefore, to the best of our knowledge, this is the first report of this invasive pathogen in the mainland Americas.

Several characteristics are predicted of invasive parasite species to enable their successful establishment into a new range area, such as to be a host generalist and to be cosmopolitan in their distribution (Ewen et al. 2012). Here we found that pSGS1 infects

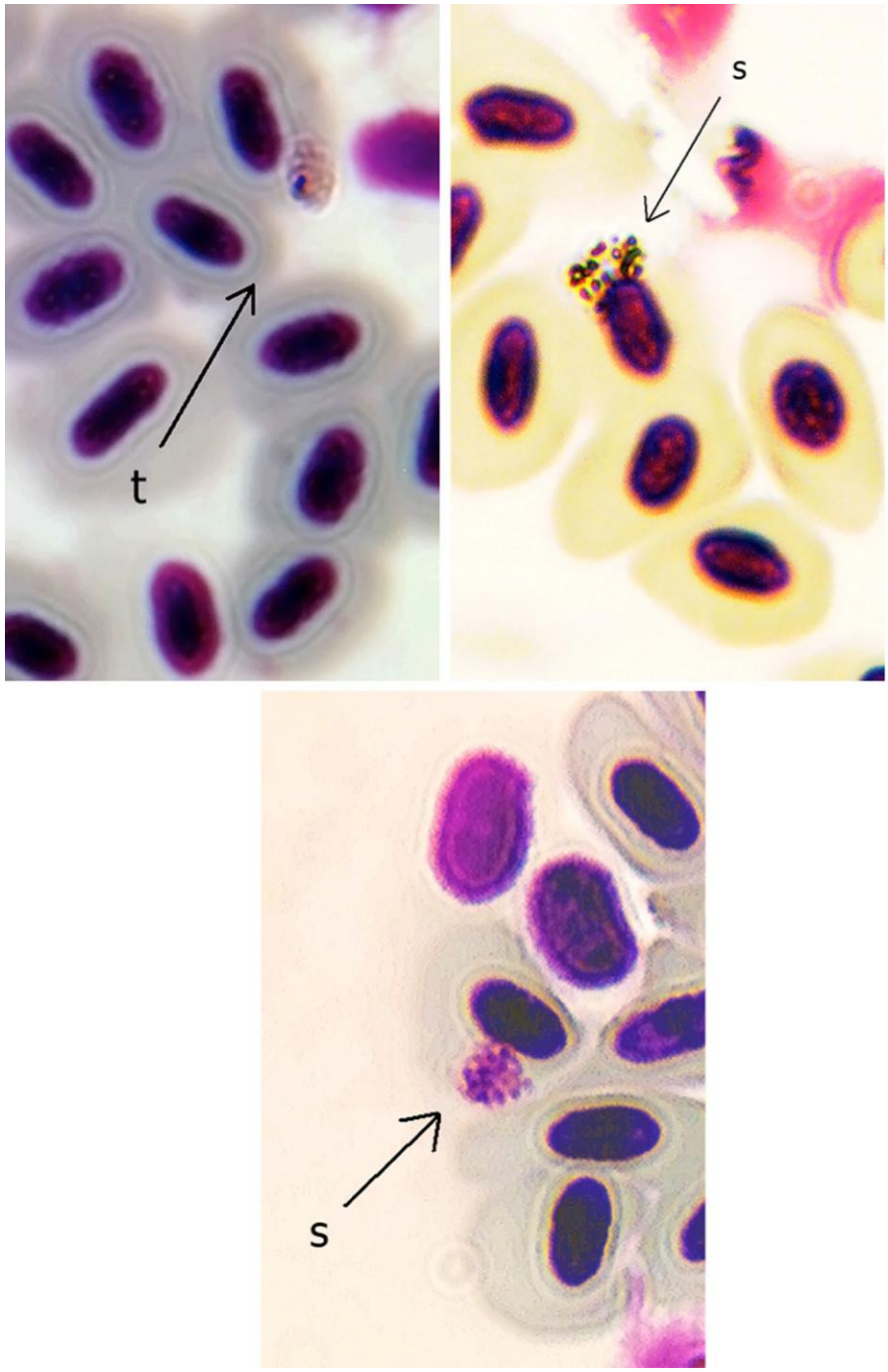

Fig. 2 Representative development structures observed in the erythrocytes from PCR-positive Plasmodium relictum SGS1 birds of our study demonstrating Plasmodium morphologies. t trophozoite, s intraerythrocytic schizont/meront

8 different host species from 2 orders of Peruvian birds, being the most host generalist parasite lineage in our study (Table 1). Moreover, pSGS1 was also the most geographically widespread parasite, being the only Plasmodium lineage infecting birds in both study areas (Table 1). These data agree with previous

Table 1 Lineage names, parasite genus (H Haemoproteus, P Plasmodium), GenBank accession numbers, prevalence of total infection, localities where have been sampled (Huan Huanuco, PV Pantanos de Villa), names of recorded hosts and number of infected and number of tested separate for each host and locality

Lineage	Genus	GenBank#	Prevalence of total infection (%)	Localities	Recorded hosts (N infected/N tested)
BAEBIC02	P	AF465555	15.15	HUAN	Zonotrichia capensis (5/23)
CHLOP01	H	JQ764618	18.18	HUAN	Zonotrichia capensis (6/23)
PHPAT01	P	EF153642	3.03	HUAN	Sayornis nigricans (1/7)
PYERY01	H	AY172842	3.03	HUAN	Carduelis magellanica (1/2)
SERCIN01	H	KF482344	3.03	HUAN	Serpophaga cinerea (1/16)
SGS1	P	AF495571	39.40	HUAN, PV	Amazilia chianogster (HUAN; 1/12), Colibri coruscans(HUAN; 1/2), Sayornis nigricans (HUAN; 2/7), Serpophaga cinerea (HUAN; 3/16), Zonotrichia capensis (HUAN; 2/23), Conirostrum cinereum (PV; 1/1), Phleocryptes melanops (PV; 1/6), Troglodytes aedon (PV; 1/3)
STTA17H	H	JN819389	9.09	HUAN, PV	Thraupis episcopus (HUAN; 2/3), Sicalis luteola (PV; 1/6)
TACHURIS01	P	KF482356	3.03	PV	Tachuris rubigastra (1/7)
TROGLODY01	P	KF482358	3.03	PV	Troglodytes aedon (1/3)
ZOCAP01	H	EF153649	3.03	HUAN	Zonotrichia capensis (1/23)

records on the host range and geographical distribution of generalist pSGS1 (Bensch et al. 2009), suggesting that pSGS1 may be a recent invader and also showing the invasive potential of this species. This invasive parasite, however, could have not become so widely established without the presence of a suitable vector. Unfortunately we are not able to confirm the origin of this emerging disease and we can only speculate about the mechanisms that may have facilitated its spread to South America. Previous reports of avian malaria outbreaks have shown that the combination of naïve native birds, infected non-native birds, and abundant competent mosquito vectors has expedited the rapid establishment and spread of avian malaria (Atkinson et al. 1995; Tompkins and Gleeson 2006). Hence, once a population of a suitable vector has been fully established, the introduction of chronic avian malaria through exotic birds (e.g. pets) and/or migratory birds may provoke an outbreak of avian malaria in endemic birds (Van Riper et al. 1986; Warner 1968). Future studies examining blood parasites from non-native birds and mosquitoes in both study areas will help to elucidate when and where infection was most likely acquired by the Peruvian native birds.

Evolutionary theory predicts that virulence of parasites will be low in hosts that have evolved with the parasite (Schmid-Hempel 2011). In contrast, exotic parasites should be highly virulent to their new hosts because the lack of evolved immunological resistance (Schmid-Hempel 2011). In this sense, invasive parasite species often cause extreme morbidity and mortality in novel hosts because naive host populations usually lacks protective immunity, resulting in high mortality. For example, as an outcome of the fatal introduction of exotic P. relictum GRW4 lineage and its competent vector in Hawaii in the 19th century, the mortality of resident birds increased up to 90 % and many native species went extinct (Atkinson et al. 1995; Beadell et al. 2006; Lapointe et al. 2012). A similar situation has been reported in New Zealand, where different exotic avian malaria parasites lineages have recently arrived in multiple independent events along with their primary vector Culex quinquefasciatus and may have an impact on New Zealand native birds (Ewen et al. 2012; Howe et al. 2012). Here we did not find that birds infected with exotic avian malaria pSGS1 had lower body mass scores than birds infected with native parasite lineages when introducing bird host species ID in the model (mean body mass

index (SD): infected with exotic malaria parasite = -1.761 (3.763); infected with endemic malaria parasite =2.226 (5.728); GLMM: estimate = -0.391, P = 0.622). However, the effects of pSGS1 on Neotropical birds could be underestimated due to the use of mist-nets, which tend to under-sample highly infected birds exhibiting high morbidity (Valkiūnas 2005). Experimental studies have shown that avian haemosporidians provoke detrimental effects on their hosts by decreasing body condition (Atkinson et al. 2000; Valkiūnas et al. 2006), which in turn may reduce adult bird survival (Valkiūnas 2005). Hence, the presence of this exotic Plasmodium lineage in birds of South America may represent a serious risk to this avifauna. Ornithological fauna from Peru represents 20 % of bird diversity of the world and more than 62 % of bird species richness of South America. Many of these species of birds are considered a priority for conservation because of their high degree of endemism or their risk of extinction (Schulenberg et al. 2010). Moreover, Peru has the second largest portion of the Amazon rain forest after the Brazilian Amazon, being one of the most biologically diverse areas on Earth. The high prevalence of this invasive parasite in the limit with Peruvian Amazon (34.6 % of the overall infection in Huanuco) should warn us about the potential threat to over one-third of all bird species in the world (Da Silva et al. 2005).

Summarizing, we show for first time the presence of invasive avian malaria P. relictum SGS1 infecting birds in the mainland Americas. Our intention is to underscore the conservation implications of invasive avian malaria and to elucidate the impact of exotic avian malaria parasites on individuals, populations and species of Neotropical birds.

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References

Atkinson CT, Woods KL, Dusek RJ et al (1995) Wildlife disease and conservation in Hawaii: pathogenicity of avian malaria (Plasmodium relictum) in experimentally infected Iiwi (Vestiaria coccinea). Parasitology 111:S59–S69

Atkinson CT, Dusek RJ, Woods KL et al (2000) Pathogenicity of avian malaria in experimentally-infected Hawaii amakihi. J Wild Dis 36:197–204

Beadell JS, Ishtiaq F, Covas R et al (2006) Global phylogeographic limits of Hawaii's avian malaria. Proc R Soc Lond B Biol Sci 273:2935–2944. doi:10.1098/rspb.2006.3671

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

Da Silva JMC, Rylands AB, Da Fonseca GAB (2005) The fate of the Amazonian areas of endemism. Conserv Biol 19:689–694. doi:10.1111/j.1523-1739.2005.00705.x

Durrant KL, Beadell JS, Ishtiaq F et al (2006) Avian malaria in South America: a comparison of temperate and tropical zones. Ornithol Monogr 60:98–111

Ewen JG, Bensch S, Blackburn TM et al (2012) Establishment of exotic parasites: the origins and characteristics of an avian malaria community in an isolated island avifauna. Ecol Lett 15:1112–1119. doi:10.1111/j.1461-0248.2012.01833.x

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

Hatcher MJ, Dick JTA, Dunn AM (2012) Disease emergence and invasions. Funct Ecol 26:1275–1287. doi:10.1111/j.1365-2435.2012.02031.x

Hellgren O, Waldenström J, Bensch S (2004) A new PCR assay for simultaneous studies of leucocytozoon, plasmodium and haemoproteus from avian blood. J Parasitol 90:797–802

Hellgren O, Peréz-Tris J, Bensch S (2009) A jack-of-all-trades and still a master of some: prevalence and host range in avian malaria and related blood parasites. Ecology 90:2840–2849. doi:10.1890/08-1059.1

Howe L, Castro IC, Schoener ER et al (2012) Malaria parasites (Plasmodium spp.) infecting introduced, native and endemic New Zealand birds. Parasitol Res 110:913–923. doi:10.1007/s00436-011-2577-z

Jakob EM, Marshall SD, Uetz GW (1996) Estimating fitness: a comparison of body condition indices. Oikos 77:61–67

Lacorte GA, Félix GMF, Pinheiro RRB et al (2013) Exploring the diversity and distribution of neotropical avian malaria parasites—a molecular survey from Southeast Brazil. PLoS One 8(3):e57770. doi:10.1371/journal.pone.0057770

Lapointe DA, Atkinson CT, Samuel MD (2012) Ecology and conservation biology of avian malaria. Ann NY Acad Sci 1249:211–226. doi:10.1111/j.1749-6632.2011.06431.x

Lowe S, Browne M, Boudjelas S et al (2000) 100 of the world's worst invasive alien species a selection from the global invasive species database. IUCN/SSC Invasive Species Specialist Group (ISSG), Auckland

Marzal A, Ricklefs RE, Valkiūnas G et al (2011) Diversity, loss, and gain of malaria parasites in a globally invasive bird. PLoS One 6(7):e21905. doi:10.1371/journal.pone.0021905

Merino S, Moreno J, Vásquez RA et al (2008) Haematozoa in forest birds from southern Chile: latitudinal gradients in prevalence and parasite lineage richness. Austral Ecol 33:329–340. doi:10.1111/j.1442-9993.2008.01820.x

Palinauskas V, Kosarev V, Shapoval A et al (2007) Comparison of mitochondrial cytochrome b lineages and morphospecies of two avian malaria parasites of the subgenera Haemamoeba and Giovannolaia (Haemosporida: plasmodiidae). Zootaxa 1626:39–50

Schmid-Hempel P (2011) Evolutionary parasitology: the integrated study of infections, immunology, ecology, and genetics. Oxford University Press, Oxford

Schulenberg TS, Stotz DF, Lane DF et al (2010) Birds of Peru, revised and updated. Princeton University Press, Princeton

Tompkins DM, Gleeson DM (2006) Relationship between avian malaria distribution and an exotic invasive mosquito in New Zealand. J Roy Soc New Zeal 36:51–62

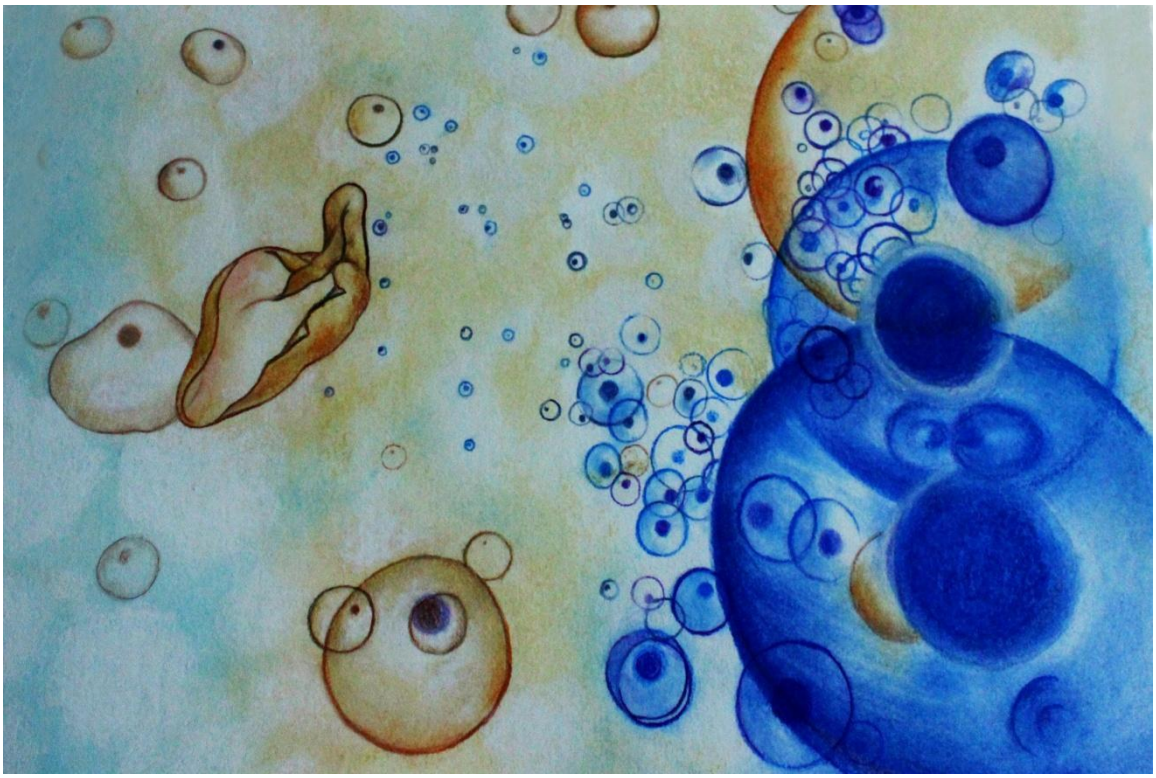
Valkiūnas G (2005) Avian malaria parasites and other haemosporidia. CRC Press, Boca Raton

Valkiūnas G, Zickus T, Shapoval AP et al (2006) Effect of Haemoproteus belopolskyi (Haemosporida: haemoproteidae) on body mass of the blackcap Sylvia atricapilla. J Parasitol 92:1123–1125

Van Riper C, van Riper SG, Goff ML et al (1986) The epizootiology and ecological significance of malaria in Hawaiian land birds. Ecol Monogr 56:327–344. doi:10.2307/1942550

Warner RE (1968) The role of introduced diseases in the extinction of the endemic Hawaiian avifauna. Condor 70:101–120

Molecular identification of the chitinase gene in *Plasmodium relictum*



IV

Deoxyribose nucleic acid structure has a novel features which will be of considerable biologic interest.

Rosalind Franklin

Los parásitos maláricos necesitan sintetizar una enzima llamada quitinasa para traspasar la membrana peritrópica, la cual es creada alrededor del estómago del mosquito, y así completar su ciclo de vida. En las especies maláricas que infectan a mamíferos, el gen de la quitinasa está formado siempre por una copia, bien una corta o bien una larga. Sin embargo, se ha detectado que en la especie malárica aviar *Plasmodium gallinaceum* están presentes ambas copias. Esto podría sugerir que las especies maláricas que afectan a aves podrían ser antecesoras de las especies maláricas que afectan a los mamíferos donde las primeras perdieron una de las dos copias, a lo largo de la evolución, dando lugar a las segundas. Por otro lado, *Plasmodium gallinaceum* no es la especie de malaria aviar más extendida y dañina en aves. Este estudio es el primero en buscar e identificar el gen de la quitinasa en uno de los parásitos de malaria aviar más prevalentes y peligrosos, *Plasmodium relictum*. Identificamos el gen de la quitinasa en dos linajes mitocondriales de *P. relictum* (SGS1 y GRW4). Comprobamos que ambos genes están compuestos por dos copias, una larga (*PrCHT1*) y otra corta (*PrCHT2*). Las diferencias genéticas en la copia larga de SGS1 y GRW4 fueron mayores que las diferencias observadas en el gen del citocromo b. Así, estos resultados podrían aclarar las relaciones filogenéticas entre especies del género *Plasmodium*. Además, debido a su elevada variabilidad genética, el gen de la quitinasa podría ser usado para estudiar la estructura poblacional de *Plasmodium* en diferentes especies de aves y regiones geográficas.

Palabras clave: malaria aviar, quitinasa, *Plasmodium relictum*, SGS1, GRW4.

MALARIA JOURNAL

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Molecular identification of the chitinase genes in Plasmodium relictum

Luz Garcia-Longoria[1*], Olof Hellgren[2] and Staffan Bensch[2]

Abstract

Background: Malaria parasites need to synthesize chitinase in order to go through the peritrophic membrane, which is created around the mosquito midgut, to complete its life cycle. In mammalian malaria species, the chitinase gene comprises either a large or a short copy. In the avian malaria parasites Plasmodium gallinaceum both copies are present, suggesting that a gene duplication in the ancestor to these extant species preceded the loss of either the long or the short copy in Plasmodium parasites of mammals. Plasmodium gallinaceum is not the most widespread and harmful parasite of birds. This study is the first to search for and identify the chitinase gene in one of the most prevalent avian malaria parasites, Plasmodium relictum.

Methods: Both copies of P. gallinaceum chitinase were used as reference sequences for primer design. Different sequences of Plasmodium spp. were used to build the phylogenetic tree of chitinase gene.

Results: The gene encoding for chitinase was identified in isolates of two mitochondrial lineages of P. relictum (SGS1 and GRW4). The chitinase found in these two lineages consists both of the long (PrCHT1) and the short (PrCHT2) copy. The genetic differences found in the long copy of the chitinase gene between SGS1 and GRW4 were higher than the difference observed for the cytochrome b gene.

Conclusion: The identification of both copies in P. relictum sheds light on the phylogenetic relationship of the chitinase gene in the genus Plasmodium. Due to its high variability, the chitinase gene could be used to study the genetic population structure in isolates from different host species and geographic regions.

Keywords: Avian malaria, Chitinase, Plasmodium relictum, SGS1, GRW4

Background

Malaria parasites have a complicated life cycle that requires several unique adaptive mechanisms that enable the parasite to successfully invade a variety of different tissues both in the vertebrate host and in the arthropod vector. Presumably as a protection against pathogens, arthropods develop a protective peritrophic membrane (PM) around their midgut after each blood meal which remains for 24 hours and then disappears [1]. The PM acts as a barrier blocking the penetration of parasites and not allowing them to spread to other organs [2]. Parasites in turn, have developed three different ways to overcome this barrier by (i) leaving the erythrocytes before the formation of the PM (as is the case Wuchereria infection) [3], (ii) persisting until the PM disappears (e.g. Leishmania) [4], or (iii) penetrating the PM (e.g. malaria parasites) [1]. The mechanism which allows malaria parasites to go through the PM of mosquitoes is well described [5-7]. These studies have shown that following the sexual process that takes place in the mosquito stomach, the ookinete has the ability to cross the PM by secreting a chitinase with characteristics of the family 18-glycohydrolases that have catalytic and substrate-binding sites that breaks down this layer [8-10]. After crossing the PM, ookinetes finally transform into oocysts which after maturing (9–11 days [11]) releases the sporozoites that move to the salivary glands where they are ready for infecting a new host (e.g. birds). Therefore, chitinase secretion has an essential role in the completion of the life cycle of malaria parasites.

The mammalian Plasmodium parasite species have a single copy of the chitinase gene but with two different structures. In the human and primate malaria parasites, Plasmodium vivax and Plasmodium knowlesi and the

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rodent parasites (Plasmodium berghei, Plasmodium yoelii and Plasmodium chabaudi) the chitinase gene is longer and contains both a catalytic domain and a chitin-binding domain; in contrast, the shorter version present in Plasmodium falciparum and Plasmodium reichenowi lacks the chitin-binding domain [12]. Remarkably, the chicken parasite Plasmodium gallinaceum has functional copies of both the long (PgCHT1) and the short (PgCHT2) chitinase gene [12] suggesting that it is a common ancestor of the mammalian Plasmodium parasites that subsequently lost either the short or the long copy of the chitinase gene [13]. The phylogenetic relationships among Plasmodium parasites infecting mammals and birds have been intensively debated over the past decades. Some studies have found support for that P. falciparum is more closely related to bird parasites than to the other mammalian malaria parasites [13,14], whereas other studies support that the mammalian parasites are forming a monophyletic clade [15,16]. Because P. gallinaceum so far is the only bird malaria parasite investigated for its chitinase genes, it is too early to establish that the occurrence of both chitinase copies is representative for bird malaria parasites in general.

Plasmodium gallinaceum has been the primary model for studies related with chitinase function in avian malaria [1,17]. However, this species is not the most common malaria parasite in birds. In fact, species belonging to the genus Plasmodium show distinct differences in their distribution and prevalence [18]. The most widespread and harmful avian malaria species is Plasmodium relictum, found to infect more than 70 different bird species, whereas P. gallinaceum has been found to infect only 4 (MalAvi data base 2013-12-02 [19]). Plasmodium relictum is one of the most generalist malaria parasite in birds and has several mitochondrial cytochrome b lineages (e.g. SGS1, GRW4, GRW11, LZFUS01) that can be found in almost all continents (MalAvi data base 2013-12-02 [19]). Full understanding of the genetic mechanisms of the infection cycle could help to gain insights into why some parasites are specialist whereas others can infect a large number of different host species.

Despite the wide distribution and harmfulness of P. relictum, no study has tried to determine either if this species has the chitinase gene, nor the number of copies it possesses. Therefore, the objectives of this study were (1) to determine whether the two most widespread lineages of P. relictum (SGS1 and GRW4) have the gene encoding for chitinase, (2) if these lineages have both copies (CHT1 and CHT2) and (3) finally determine the genetic variability of chitinase genes between the lineages SGS1 and GRW4.

Methods

Chitinase identification and sequencing

Geneious 6.1. software primer design tool was used to create primers for amplification of overlapping partial regions within the catalytic domain of both copies of the P. gallinaceum chitinase genes (long: AF064079; short: AY842482). Each of the copies of the chitinase gene was first aligned to the sequences of all other available mammalian Plasmodium parasites in order to identify conserved regions. Figure 1 shows the position of the primers in both fragments of P. gallinaceum and the primer sequences are given in Table 1.

Two samples from previous experimental infections with P. relictum, the cytochrome b lineage SGS1 from crossbills [20] and GRW04 from great reed warblers [21], were used as DNA template. Total genomic DNA from the avian blood samples was extracted by standard ammonium acetate protocol [22]. All samples were screened for chitinase using a nested PCR method for chitinase genes with primers as in Table 1. For both steps, PCR reactions were set up in total volumes of 25 μl, containing 15.4 μl of ddH2O, 1.5 μl of MgCl2 (25 mM), 2.5 μl dNTP (10 mM), 2.5 μl 10x Buffer, 1 μl of each primer (10 μM), 0.1 of Taq polymerase and 1 μl of each sample (25 ng DNA/μl). The PCR temperature profile was 95°C for 2 min followed by 25 or 35 cycles of 95°C for 30 sec, annealing temperature according to Table 1 for 30 sec and 72°C for 30 sec and terminated by a step of 72°C for 10 min. For the SGS1 isolate we used an additional set of primers (PgCHT1_F3, PgCHT1_R3, PgCHT2_F3, PgCHT2R4) to amplify a region 3' to the fragment obtained with the nested protocol. Positive amplifications were precipitated and sequenced using a dye terminator cycling sequencing (big dye) kit and loaded on an ABI PRISM™ 3100 sequencing robot (Applied Biosystems. Florida. USA).

Phylogenetic analysis

Sequences from P. relictum were aligned with the available chitinase gene sequences from Plasmodium spp. (P. gallinaceum CHT1: AF064079; CHT2: AY842482; P. berghei CHT1: AJ305256; P. yoelii CHT1: AB106898; P. knowlesi CHT1: XM002257469; P. vivax CHT1: AB106896; P. falciparum CHT1: AF127445; P. reichenowi CHT1: AY842483) using Geneious translation alignment tool. The quality of the alignment was checked by manual inspection. The combined phylogenetic tree for the two copies was constructed in the programme MEGA 5.2 and using a Maximum Likelihood model. Bootstrap values were used in order to obtain a consensus phylogeny using 200 iterations.

Results

Both lineages of P. relictum (SGS1 and GRW4) had both gene copies encoding for chitinase. The obtained sequences for the lineage SGS1 and GRW4 were 852 bp and 339 bp for the long copy (GenBank accession number KJ452165, KJ452167) and 845 bp and 393 bp for the short copy

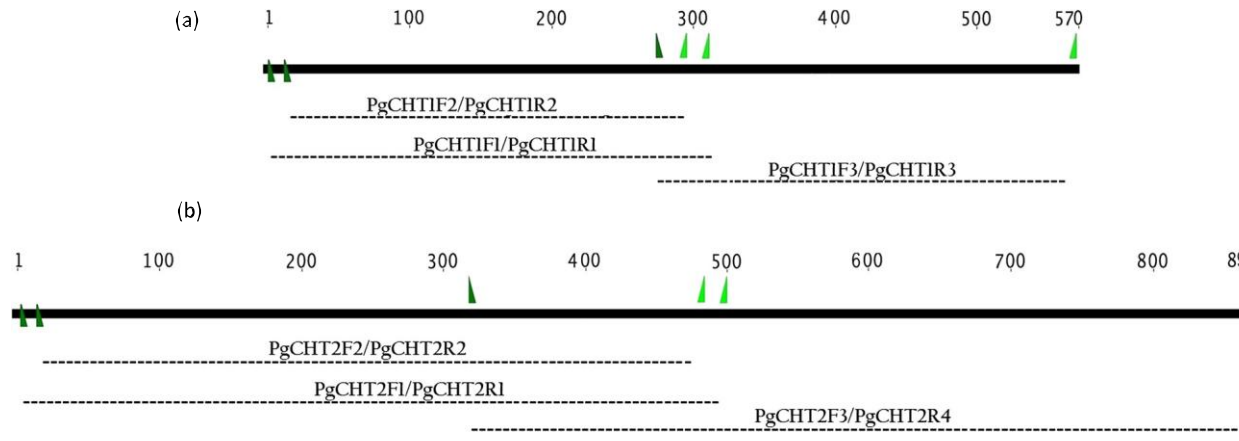

Figure 1 Primer sites (Forward: dark green arrows, Reverse: light green arrows) along the cht1 (a) and cht2 (b). Amplified fragments are represented by dashed lines.

(GenBank accession number KJ452166, KJ452168). Because the obtained sequences from the GRW4 isolate were short only the data from the SGS1 isolate was used for the phylogenetic analyses. These two regions do not completely overlap (Figure 2). As a result, both sequences were trimmed to only cover the shared sites resulting in a combined alignment covering a region of 802 nucleotides. The phylogenetic analyses found strong support for the separation of the long and the short copies, both being present in the SGS1 isolate of P. relictum (Figure 3).

The nucleotide (and amino acid) distances were compared between P. gallinaceum and P. relictum for both copies using a Pairwise distance matrix. For the long copy a distance of 10.0% was found (9.2%) between SGS1 and P. gallinaceum. For the short copy, a distance of 11.0% (10.6%) was found between SGS1 and P. gallinaceum. Over the regions for which data of P. relictum are available from both isolates of P. relictum, SGS1 and GRW4 differed by 1.5% (0.8%) for the short copy and 4.1% (3.6%) for the long copy.

Discussion

The chitinase gene can consist of one or two copies [9], a long and a short one. Previous studies have established that some malaria parasites only have one copy (e.g. P. falciparum [10,23] and P. berghei [24]) while only P. gallinaceum has both variants [9]. Molecular results showed that P. relictum has both copies encoding for chitinase (PrCHT1 and PrCHT2). Plasmodium relictum is as far as it is known, the second malaria parasite demonstrated to have both copies. As P. gallinaceum and P. relictum are quite distantly related among the Plasmodium parasites infecting birds [15,16] suggests that the presence of two chitinase gene copies is widespread among the bird

Table 1 Annealing temperature for all the primers used

Primer		Seq (as ordered)	Annealing temp. (°C)
PgCHT1_F	Forward	5'-ATGATAGAAAATCACCAAGACAAATTTTAGA-3'	50
PgCHT1_R	Reverse	5'-GGTTCCCAGTCAATATCTACACCA-3'	50
PgCHT1_F2	Forward	5'-TAGAGGAATACAAAAGAAGGAAACAAGG −3'	50
PgCHT1_R2	Reverse	5'-CAGTCAATATCTACACCATCTAAATCA −3'	50
PgCHT2_F	Forward	5'-ATTCAAGGTTATTATCCATCATGGGT-3'	53
PgCHT2_R	Reverse	5'-GAAATCCTATACAGCTCAAAGCTCC −3'	53
PgCHT2_F2	Forward	5'-GGGTGTCATATAATCATAATATGAAAGA −3'	53
PgCHT2_R2	Reverse	5'-GACATTGATATTAATTTATCCTCACACA −3'	53
PgCHT1_F3	Forward	5'-AATGACTTTGATTTAGATGGTGTAGAT-3'	55
PgCHT1_R3	Reverse	5'-TAATTGTTCTTTCATAAATAAATGCCA −3'	55
PgCHT2_F3	Forward	5'-ATGAACCCAATGGATCGTTTGATG −3'	58
PgCHT2_R4	Reverse	5'-TAAATTATTAGACAAAGACCACAATCC −3'	58

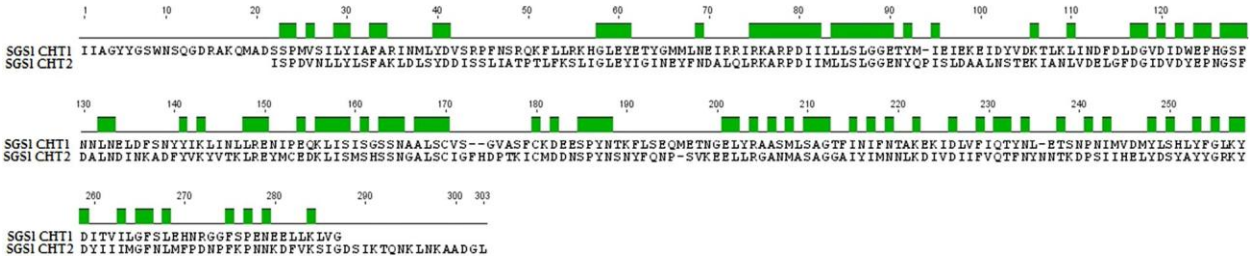

Figure 2 Overlap of the long (PrCHT1) and short copy (PrCHT2) sequenced in the mitochondrial lineage SGS1 of P. relictum. The aminoacid sequence is shown in order to illustrate the overlap (green areas).

Plasmodium parasites. Hence, avian malaria parasites are, to date, the only parasites with both copies. Li et al. [12] suggested that avian malaria parasites could be the ancestor for the chitinase gene in malaria parasites of primates and rodents. Thus, given the current phylogenetic hypothesis, it can be assumed that mammalian parasites evolved from an avian parasite that carried two copies of the chitinase gene.

The bar-coding gene for molecular identification of Plasmodium parasites of birds is the cytochrome b gene [25-27]. When a genetic difference between lineages exceeds 5% this is often followed by distinct morphological differentiation which allows for identification of morphological defined species [26]. Obviously, differences are lower when lineages within the same morphological defined species are compared. The MalAvi data base [19] shows that the genetic variability in the cytochrome b between SGS1 and GRW4 is 1.8% (9 nucleotides different in 480 bp). However, the present study shows that the genetic variability between SGS1 and GRW4 in the chitinase gene was much higher, 4.1% (14 different nucleotides in 339 bp). Moreover, the genetic distance in the cytochrome b between P. relictum and P. gallinaceum is 6.9% (29 nucleotides different in 480 bp). The results of this study shows that the genetic distance between P. relictum and P. gallinaceum in the short copy was 13.1% (44 different nucleotides in 339 bp). Previous studies have identified some nuclear genes with a high variability in P. relictum, for instance the msp1 gene [28], that can be used for epidemiological studies of the malaria parasite. In the same way, the chitinase gene could be a good candidate and complement for studies of genetic population structure of the parasites.

In conclusion, the present study demonstrates that the most widespread and harmful avian malaria parasite, P. relictum, have the gene encoding for chitinase. In accordance with previous studies on avian malaria (i.e. P. gallinaceum), the present study demonstrates that occurrence of both copies (PrCHT1 and PrCHT2) seems to be widespread across avian Plasmodium species. Additionally, the present study demonstrates that the genetic variability of the chitinase gene was high between the two analysed lineages of P. relictum (SGS1 and GRW4).

To determine the phylogenetic relationship between the chitinase gene in malaria parasites, future studies could search for the number of fragments in other species of haemosporidian parasites and most importantly in the genera closely related to Plasmodium that are

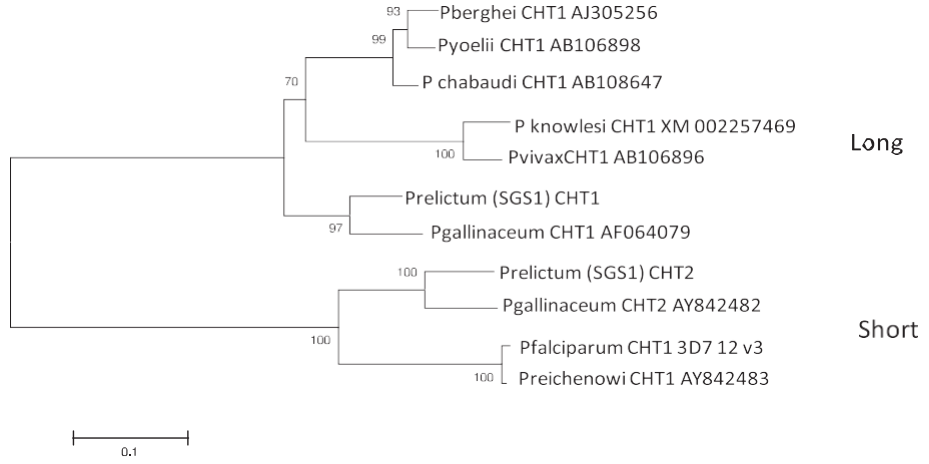

Figure 3 Maximum likelihood consensus phylogeny using midpoint rooting of the translated CHT1 (long copy) gene and CHT2 (short copy) from 10 different malaria species. Numbers in branches represent bootstrap values based on 200 iterations.

transmitted by vectors of other dipteran families than Culicidae. Another interesting approach would be to analyse the chitinase gene in parasites isolated from a wide range of bird species with a high prevalence of P. relictum and different habitat uses, looking at the gene variability in P. relictum.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

LG carried out the molecular work on the chitinase gene and drafted the manuscript, OH provided the samples analysed. SB designed the study and interpreted the data. OH and SB made important contribution to drafting the manuscript. All authors read and approved the final manuscript.

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References

1. Sieber KP, Huber M, Kaslow D, Banks SM, Torii M, Aikawa M, Miller LH: The peritrophic membrane as a barrier: its penetration by Plasmodium gallinaceum and the effect of a monoclonal antibody to ookinetes. Exp Parasitol 1991, 72:145–156.
2. Ghosh A, Edwards MJ, Jacobs-Lorena M: The journey of the malaria parasite in the mosquito: hopes for the new century. Parasitol Today 2000, 16:196–201.
3. Perrone JB, Spielman A: Microfilarial perforation of the midgut of a mosquito. J Parasitol 1986, 72:723–727.
4. Schlein Y, Jacobson RL, Messer G: Leishmania infections damage the feeding mechanism of the sandfly vector and implement parasite transmission by bite. Proc Natl Acad Sci U S A 1992, 89:9944–9948.
5. Tsai YL, Hayward RE, Langer RC, Fidock DA, Vinetz JM: Disruption of Plasmodium falciparum chitinase markedly impairs parasite invasion of mosquito midgut. Infect Immun 2001, 69:4048–4054.
6. Li F, Templeton TJ, Popov V, Comer JE, Tsuboi T, Torii M, Vinetz JM: Plasmodium ookinete-secreted proteins secreted through a common micronemal pathway are targets of blocking malaria transmission. J Biol Chem 2004, 279:26635–26644.
7. Kadota K, Ishino T, Matsuyama T, Chinzei Y, Yuda M: Essential role of membrane-attack protein in malarial transmission to mosquito host. Proc Natl Acad Sci U S A 2004, 101:16310–16315.
8. Huber M, Cabib E, Miller LH: Malaria parasite chitinase and penetration of the mosquito peritrophic membrane. Proc Natl Acad Sci U S A 1991, 88:2807–2810.
9. Vinetz JM, Valenzuela JG, Specht CA, Aravind L, Langer RC, Ribeiro JM, Kaslow DC: Chitinases of the avian malaria parasite Plasmodium gallinaceum, a class of enzymes necessary for parasite invasion of the mosquito midgut. J Biol Chem 2000, 275:10331–10341.
10. Vinetz JM, Dave SK, Specht CA, Brameld KA, Xu B, Hayward R, Fidock DA: The chitinase PfCHT1 from the human malaria parasite Plasmodium falciparum lacks proenzyme and chitin-binding domains and displays unique substrate preferences. Proc Natl Acad Sci U S A 1999, 96:14061–14066.
11. Boysen KE, Matuschewski K: Arrested oocyst maturation in Plasmodium parasites lacking type II NADH: Ubiquinone Dehydrogenase. J Biol Chem 2011, 286:32661–32671.
12. Li F, Patra KP, Vinetz JM: An anti-chitinase malaria transmission-blocking single-chain antibody as an effector molecule for creating a Plasmodium falciparum-refractory mosquito. J Infect Dis 2005, 192:878–887.
13. Waters AP, Higgins DG, McCutchan TF: Plasmodium falciparum appears to have arisen as a result of lateral transfer between avian and human hosts. Proc Natl Acad Sci U S A 1991, 88:3140–3144.
14. Pick C, Ebersberger I, Spielmann T, Bruchhaus I, Burmester T: Phylogenomic analyses of malaria parasites and evolution of their exported proteins. BMC Evol Biol 2011, 11:167.
15. Perkins SL, Schall JJ: A molecular phylogeny of malarial parasites recovered from cytochrome b gene sequences. J Parasitol 2002, 88:972–978.
16. Martinsen ES, Perkins SL, Schall JJ: A three-genome phylogeny of malaria parasites (Plasmodium and closely related genera): evolution of life-history traits and host switches. Mol Phylogenet Evol 2008, 47:261–273.
17. Shahabuddin M, Toyoshima T, Aikawa M, Kaslow DC: Transmission-blocking activity of a chitinase inhibitor and activation of malarial parasite chitinase by mosquito protease. Proc Natl Acad Sci U S A 1993, 90:4266–4270.
18. Valkiūnas G: Avian Malaria Parasites and Other Haemosporidia. Boca Raton: CRC Press; 2005.
19. Bensch S, Hellgren O, Pérez-Tris J: MalAvi: a public database of malaria parasites and related haemosporidians in avian hosts based on mitochondrial cytochrome b lineages. Mol Ecol Resour 2009, 9:1353–1358.
20. Palinauskas V, Valkiūnas G, Bolshakov CV, Bensch S: Plasmodium relictum (lineage P-SGS1): effects on experimentally infected passerine birds. Exp Parasitol 2008, 120:372–380.
21. Zehtindjiev P, Ilieva M, Westerdahl H, Hansson B, Valkiūnas G, Bensch S: Dynamics of parasitemia of malaria parasites in a naturally and experimentally infected migratory songbird, the great reed warbler Acrocephalus arundinaceus. Exp Parasitol 2008, 119:99–110.
22. Richardson DS, Jury FL, Blaakmeer K, Komdeur J, Burke T: Parentage assignment and extra-group paternity in a cooperative breeder: the Seychelles warbler (Acrocephalus sechellensis). Mol Ecol 2001, 10:2263–2273.
23. Langer RC, Vinetz JM: Plasmodium ookinete-secreted chitinase and parasite penetration of the mosquito peritrophic matrix. Trends Parasitol 2001, 17:269–272.
24. Dessens JT, Mendoza J, Claudianos C, Vinetz JM, Khater E, Hassard S, Ranawaka GR, Sinden RE: Knockout of the rodent malaria parasite chitinase pbCHT1 reduces infectivity to mosquitoes. Infect Immun 2001, 69:4041–4047.
25. Krizanauskiene A, Hellgren O, Kosarev V, Sokolov L, Bensch S, Valkiūnas G: Variation in host specificity between species of avian hemosporidian parasites: evidence from parasite morphology and cytochrome B gene sequences. J Parasitol 2006, 92:1319–1324.
26. Hellgren O, Krizanauskiene A, Valkiūnas G, Bensch S: Diversity and phylogeny of mitochondrial cytochrome B lineages from six morphospecies of avian Haemoproteus (Haemosporida: Haemoproteidae). J Parasitol 2007, 93:889–896.
27. Omori S, Sato Y, Isobe T, Yukawa M, Murata K: Complete nucleotide sequences of the mitochondrial genomes of two avian malaria protozoa, Plasmodium gallinaceum and Plasmodium juxtanucleare. Parasitol Res 2007, 100:661–664.
28. Hellgren O, Kutzer M, Bensch S, Valkiūnas G, Palinauskas V: Identification and characterization of the merozoite surface protein 1 (msp1) gene in a host-generalist avian malaria parasite, Plasmodium relictum (lineages SGS1 and GRW4) with the use of blood transcriptome. Malar J 2013, 12:381.

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Detecting transmission areas of malaria parasites in a migratory bird species

V

Never agree to *crawl* if you want to soar.

Hellen Keller

SGS1, uno de los linages de *Plasmodium relictum*, tiene una transmisión activa en zonas tropicales de África y en áreas templadas de Europa. La detección de nuevas secuencias nucleares de este linaje (basados en diversidades alélicas de MPS1) ha proporcionado nuevas evidencias sobre la distribución geográfica y las áreas de transmisión de estos alelos. Por ejemplo, los alelos del gen MSP1 que se transmiten en África difieren de los transmitidos en Europa, sugiriendo la existencia de dos poblaciones de SGS1. Sin embargo, hasta la fecha, ningún estudio ha analizado la distribución de alelos africanos y europeos en aves migratorias. Con ese objetivo, investigamos si aviones comunes juveniles se infectaban en Europa antes de su primera migración a África. Analizamos la diversidad alélica del gen MSP1 en aviones adultos y juveniles infectados con SGS1. Encontramos que los juveniles se infectaban con SGS1 durante sus primeras semanas de vida, confirmando así una transmisión activa de SGS1 en aviones nacidos en Europa Además, encontramos que todos los juveniles y la mayoría de los adultos estaban infectados con un alelo de MSP1 transmitido en Europa mientras que dos aviones adultos estaban infectados, respectivamente, por dos alelos de MSP1 transmitido en África. Estos resultados sugieren que los aviones comunes están expuestos a diferentes poblaciones de *P. relictum* en sus cuarteles de invernada y en los de cría.

Palabras clave: *Plasmodium relictum*, avión común, *Delichon urbica,* MSP1, malaria aviar.

Detecting transmission areas of malaria parasites in a migratory bird species

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ABSTRACT

Cytochrome b lineage of *Plasmodium relictum*, SGS1, is known to have active transmission in tropical Africa and temperate regions of Europe. Nuclear sequence data from isolates infected with SGS1 (based on MSP1 allelic diversity) have provided new insights on the distribution and transmission areas of these allelic variants. For example, MSP1 alleles transmitted in Africa differ from those transmitted in Europe, suggesting the existence of two populations of SGS1 lineages. However, no study has analysed the distribution of African and European transmitted alleles in Afro-Palearctic migratory birds. With this aim, we investigated whether juvenile house martins become infected in Europe before their first migration to Africa. We explored the MSP1 allelic diversity of *P. relictum* in adult and juvenile house martins. We found that juvenile house martins were infected with SGS1 during their first weeks of life, confirming active transmission of SGS1 to house martins in Europe. Moreover, we found that all the juveniles and most of adult house martins were infected with one European transmitted MSP1 allele, whereas two adult birds were infected with two African transmitted MSP1 alleles. These findings suggest that house martins are exposed to different strains of *P. relictum* in their winter and breeding quarters.

Keywords: avian malaria; *Delichon urbica*; juveniles; MSP1; *Plasmodium relictum*.

INTRODUCTION

Climate changes are likely to affect the biology and ecology of vectors with consequences on changing transmission areas of blood parasites such as malaria and other haemosporidians (Githeko *et al.*, 2000). However, areas of transmission of different vector-borne diseases remain a key knowledge gap in our understanding of these pathogens. Parasites can regulate their host populations by reducing the fecundity or the survival of their host population, but our current knowledge of population regulation of hosts by parasites is still limited (see reviews in Møller, 2005; Schmid-Hempel, 2011). In the case of haemosporidian parasites, most of the mortalities of infected birds normally occur during the acute phase of the parasite infection, which usually happens several days after the parasite transmission (Valkiūnas, 2005). Juvenile birds are especially susceptible for infection because they are immunologically naïve, which may drive to population decline (Samuel *et al.*, 2011). Hence, it becomes essential to identify the regions where vector-borne diseases are transmitted in order to study the host population dynamics and to recognize future changes in environmental conditions that may potentially influence the transmission areas.

Haemosporidians are among the most well studied blood parasites of reptiles, mammals and birds (Valkiūnas, 2005). Avian *Plasmodium* species show a cosmopolitan distribution, being found in all continents except Antarctica (Valkiūnas, 2005). To date, more than 50 morphospecies of avian malaria parasites of the genus *Plasmodium* have been described worldwide (Valkiūnas, 2005; Palinauskas *et al.*, 2007). *Plasmodium relictum* is one of the most widespread and harmful parasite species of avian malaria, being responsible for mass mortality, population declines and even extinctions of many bird species (Van Riper III *et al.*, 1986; Valkiūnas, 2005). For all these reasons and due to its devastating effects, the International Union for Conservation of Nature (IUCN) classifies *P. relictum* as one of the worst invasive species in the world (Lowe *et al.*, 2000). Therefore, it becomes essential to identify the geographical distribution of *P. relictum* lineages and to assess their infection prevalence in birds in order to develop appropriate management strategies to promote biodiversity conservation policies worldwide.

With the use of mtDNA cytochrome b gene (cyt b) to barcode the parasites more than 500 avian *Plasmodium* parasite lineages have been (MalAvi database 2015-01-15) (Bensch *et al.*, 2009). Moreover, four different cyt b lineages have been described within the morphologically described species of *P. relictum* (Palinauskas *et al.*, 2007; Valkiūnas *et al.*, 2007; Ilgunas *et al.*, 2013; Kazlauskiene *et al.*, 2013). Two of the *P. relictum* cyt b

lineages (SGS1 and GRW4) are some of the most abundant and geographically widespread of all bird *Plasmodium* lineages. Both lineages are host generalists infecting 95 species of 28 families (SGS1) and 60 species in 19 families (GRW4) (MalAvi database 2015-01-15) (Bensch *et al.*, 2009), respectively. The lineages SGS1 and GRW4 exhibit different transmission areas (Hellgren *et al.*, 2007), with GRW4 being transmitted in New Zealand, Africa, Asia and America (Beadell *et al.*, 2006; Marzal *et al.*, 2011), whereas SGS1 shows a widespread distribution in Europe, Africa and Asia (Palinauskas *et al.*, 2007). Recently, SGS1 was also detected in Oceania (Howe *et al.*, 2012) and South America (Marzal *et al.*, 2015). In consequence, SGS1 was suggested to be one of the few *Plasmodium* lineages with active transmission in both tropical Africa and temperate regions of Asia and Europe (Hellgren *et al.*, 2007).

Investigations based on multiple nuclear loci of *P. relictum* have provided new insights into allelic variation, geographical structure and parasite transmission (Hellgren *et al.*, 2013). The merozoite surface protein 1 (MSP1) is a gene which shows a high variability (Miller *et al.*, 1993) and encodes a protein involved in the attachment of the malaria parasite to the red blood cell (Gerold *et al.*, 1996). Because its high variability this gene is a good candidate for investigation of the population structure and phylogeography of malaria lineages. For example, the SGS1 lineage transmitted in tropical Africa have a different set of MSP1 alleles compared to those transmitted in Europe, suggesting the existence of separate SGS1 populations along the European-African migratory flyways (Hellgren *et al.*, 2015). This pattern implies the existence of transmission barriers (e.g. vector communities or abiotic factors) limiting transmission between regions, but further studies are required to confirm this geographical distribution.

The house martin is a migratory species with a high fidelity to their area of hatching and nesting (Cramp and Perrins, 1994; Lope and Silva, 1998). This species migrates from Africa to Europe for breeding. Once the breeding is completed, adult house martins and new-born individuals migrate back to their African wintering quarters (Cramp and Perrins, 1994; Tumer and Rose, 1989). Previous studies in different localities of Europe and Northern-Africa have found haemosporidian infections in more than 70 % of adults (Marzal *et al.*, 2008; Piersma and van der Velde, 2012; Marzal *et al.*, 2013a, 2013b; Van Rooyen *et al.*, 2014). Additionally, different *P. relictum* cyt b lineages such as SGS1, GRW4 and GRW11 have been found infecting adult house martins in these populations (Marzal *et al.*, 2008; Piersma and van der Velde, 2012; Marzal *et al.*, 2013b; van Rooyen *et al.*, 2014). These blood parasite

infections are supposed to be transmitted on the African wintering grounds or during migration. This assumption is based on the absence of haemosporidian infection in the single study analyzing haemosporidian infections in 112 fledgling and juvenile house martins before their first migration (Piersma and van der Velde, 2012). However, the confirmation of transmission areas of haemosporidian parasites in house martins requires further investigation. Therefore, the first goal of our study was to determine whether haemosporidian transmission in house martins do occur at European sites by sampling juvenile birds. Additionally, the second objective of this study was to analyse the MSP1 alleles in *P. relictum* lineages infecting adult and juvenile house martins in order to identify their potential areas of transmission.

MATERIAL AND METHODS

Study site and collecting samples

The study was carried out in a colony of house martins in the surroundings of Badajoz (38° 50’ N, 6° 59’ W), southwest Spain, during a 6-year period (2006–2012) as part of a longer study. For the present study we captured 422 house martins, 310 of them were classified as juveniles according to the morphological characteristics established by Svensson *et al.*, (2009) and Lope (1986). Most of individuals were caught in July, at the end of their breeding season (Pajuelo *et al.*, 1992). All birds were individually identified with numbered metal rings. One microcapillary of blood (70 µl) was obtained from the brachial vein of each individual and stored in 500 µl of SET buffer (0.15 M NaCl, 0.05 Tris, 0.001 M EDTA, pH 8.0) until DNA extraction.

Molecular detection of blood parasite infections

Haemosporidian parasites (*Plasmodium* spp.) were detected from blood samples using molecular methods (Bensch *et al.*, 2000; Waldenström *et al.*, 2004). DNA from the avian blood samples were extracted in the lab using the standard phenol/chloroform/isoamylalcohol method (Sambrook *et al.*, 2002). Diluted genomic DNA (25 ng/µl) was used as a template in a polymerase chain reaction (PCR) assay for detection of the parasites using nested PCR-protocols described by Waldenström *et al.*, (2004). The amplification was evaluated by running 2.5 µl of the final PCR on a 2% agarose gel. All PCR experiments contained one negative control for every eight samples. In the very few cases of negative controls showing signs of amplification (never more than faint bands in agarose gels), the whole PCR-batch was run again to make sure that all positives were true. All positive amplifications were precipitated and sequenced in order to

identify the species and lineage in each infection. The obtained sequences were edited, aligned and compared in a sequenced matrix using the program Bioedit (Hall, 1999). We selected SGS1 and GRW4 infected house martins for further analyses of the MSP1 gene (270 nucleotides, block 14) and detect the MSP1 allele following the protocol described by Hellgren *et al.,* (2013) and using the primers MSP1_3F, MSP1_3R, MSP1_3FN and MSP1_3RN.

Phylogenetic reconstruction

The obtained sequences were edited, aligned against the SGS1_MSP1 gene (KC969175) and compared in a sequence identity matrix using the program BioEdit (Hall, 1999). The quality of the alignment was checked by manual inspection. Genetic differences between the MSP1 alleles were calculated using a Jukes-Cantor model as implemented in MEGA 5.2. We used MEGA 5.2 for phylogenetic reconstruction of the MSP1 alleles where the homolog sequence of *P. gallinaceum* (AJ809338.1) was used as an out-group. The phylogenetic tree for all the alleles found was constructed in the programme MEGA 5.2 and using a Maximum Likelihood model. Bootstrap values were used in order to obtain a consensus phylogeny using 200 iterations.

RESULTS

Prevalence of infection and genetic parasite diversity (Cyt b gene analyses)

We analyzed 422 blood samples from adult (*N* = 112) and juvenile (*N* = 310) house martins in search for haemosporidian parasites. Among adults 80 (71%) individuals were infected with haemosporidian parasites. In juveniles only three where found to be infected (0.96%).

Of the 80 infected adult birds, 20 % were infected with *Plasmodium* spp. and 80 % were infected with *Haemoproteus* spp. We found five different blood parasite lineages infecting adult house martins, of which three were of the genus *Haemoproteus* (DELURB1: 32 infected birds; DELURB2: 29 infected birds; DELURB3: 3 infected bird), and two of them from the genus *Plasmodium* (SGS1: 15 infected birds; GRW4: 1 infected bird). The three infected juveniles were all infected with the *P. relictum* lineage SGS1.

Genetic parasite diversity (MSP1 gene analyses)

All the samples infected with *P. relictum* lineages (*N* = 19; 16 adults and 3 juveniles) were selected for further molecular analyses. From each sample, we obtained a 268 bp MSP1_ b14 fragment of high quality. We used a SGS1_MSP1 gene (KC969175) in order to confirm the amplification of the MSP1 block

14 (MSP1_b14). Within adults, 14 out of 16 individuals showed the allele Pr2 (SGS1), whereas one individual was infected with Pr1 (SGS1) and the other one was infected with Pr4 (GRW4) (Figure 1). Moreover, we found the same allele (Pr2; SGS1) in all the juvenile house martins infected with malaria (Figure 1).

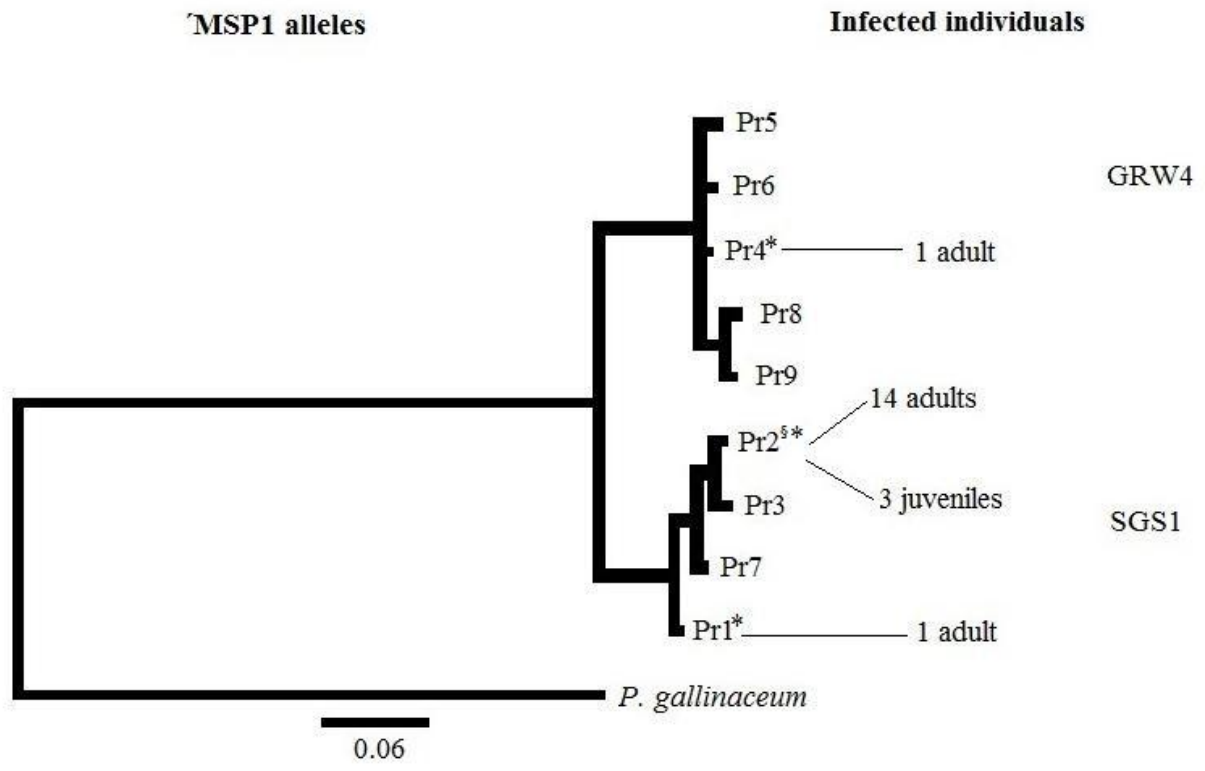

Figure 1. Phylogenetic relationship between all the MSP1_b14 alleles detected to date (Hellgren et al. 2015) and number of individuals (adults or juveniles) infected by these alleles. * and § represent confirmed active transmission in Africa and Europe, respectively (Hellgren et al. 2015).

DISCUSSION

In this study we analysed blood samples from adults and juvenile house martins in search for haemosporidian parasites. We showed, for the first time, that juvenile house martins become infected with *Plasmodium* parasites already before their first migration to Africa, thus confirming that active transmission of *Plasmodium* spp. to house martins occur in Europe. By analyzing the MSP1 alleles in *P. relictum* lineages, we were able to get a more detailed view of the likely transmission areas

for the infections found in the adult birds. Below, we will discuss the biological meaning of these results in detail.

House martins have been used in several studies to analyse life-history consequences of haemosporidian infections (e.g. Piersma and van der Velde, 2012; Marzal *et al.*, 2013b; Marzal *et al.*, 2013a; Van Rooyen *et al.*, 2014). In our study, we show that 70 % of adults were infected with haemosporidian parasites. This prevalence is similar to what has been reported from previous studies in this house martin population (Marzal *et al.*, 2008, 2013a; b). Moreover, the prevalence of haemosporidian parasites among adult house martins greately exceed the prevalence in juveniles. This difference may be explained by a higher mortality of young individuals during the infection before these being captured due to their näive immune system (Sol *et al.*, 2003) and / or the maintenance of haemosporidian infection in infected birds that survived the acute phase of infection (Valkiūnas, 2005). Alternatively, the juveniles may not yet have been exposed to infections or only recently been infected and in the phase when *Plasmodium* cryptozoites are developing in reticuloendothelial cells and therefore absent in the blood stream (Valkiūnas ,2005). Migratory birds are exposed to at least two different parasite communities during their annual cycle. According to this, migratory species such as house martins could get the blood parasite infections during their breeding season in Europe and / or in their African winter quarters and at stop-over sites. Moreover, the parasites could be transported within the migratory bird and be able to be infect a resident bird in the new area. However, Hellgren *et al.*, (2007) investigated the degree of geographical shifts of transmission of 259 haemosporidian parasite lineages. They showed that most of the parasite lineages are restricted to a specific area and thus dispersing from one biogeographical zone to another is a rare and slow evolutionary process. In agreement with these findings, all the recent studies exploring the prevalence and genetic diversity of haemosporidian parasites in house martins assumed that haemosporidian parasites are only actively transmitted on the African wintering grounds or during migration (Marzal *et al.*, 2008; Piersma and van der Velde, 2012; Marzal *et al.*, 2013a, 2013b; Van Rooyen *et al.*, 2014). This assumption was supported with the results showed by Piersma and van der Velde, (2012) in a population of house martins in the Netherlands, where none of the analyzed juveniles were infected with haemosporidian parasites. But contrary to this statement, here we have detected the presence of malaria parasites in juvenile house martins. As far as we know, this is the first study revealing an active transmission of *Plasmodium* parasites in house martins in

Europe. These findings give rise to new questions about the transmission areas of malaria parasites in this migratory bird species.

The use of data on the distribution of nuclear MSP1 alleles across the cyt. b lineages isolates may facilitate the investigation on the distribution of these malaria parasites across geographical regions (Hellgren *et al.,* 2013; Hellgren *et al.,* 2015). A recent study have explored the global phylogeography of the *P. relictum* based on MSP1 allelic diversity, showing several different MSP1 alleles within the cyt b lineages of SGS1 and GRW4. In this study we found two MSP1 alleles that is thought to be transmitted in tropical Africa Pr1 (SGS1) and Pr4 (GRW4) as well as one allele that is thought to be confined to temperate regions, Pr2 (SGS1) (Hellgren *et al.,* 2015). This pattern suggests the existance of barriers limiting the tramission areas of these parasites. In the present study we have shown that most of the adult birds infected with SGS1, as well as all the infected juveniles, carried the European transmitted MSP1 allele, Pr2 . Only one adult house martins out of 15 birds was infected with a tropical transmitted SGS1 allele (MSP1 allele Pr1), while the other one was infected with GRW4 (Pr4) which is known to have tropical transmission in the old world. These results indicate the existence of two different areas of transmission of malaria parasites for house martins population: one in the African winter quarters (Pr1 and Pr4), and the other one in the European breeding quarters (Pr2). Moreover, the high number of house martins infected with the MSP1 allele Pr2 compared to the low number infected with the MSP1 African-transmitted alleles (Pr1 and Pr4) would suggest that most of the malaria transmission takes place in Europe during the breeding season. This finding agrees with previous studies indicating that haemosporidian parasites are usually transmitted during breeding season in temperate regions, because biotic and abiotic factors are optimal for the transmission of vector-borne diseases such as malaria (Valkiūnas, 2005; Cosgrove *et al.,* 2008; *but also see* Dunn *et al.,* 2014). However, we cannot exclude that a high number of infections may also occur in tropical Africa during the winter or at stop-over sites, but that such infected individuals may not reach their European breeding quarters because they die during migration. In this line, several studies have shown that blood parasites may increase mortality in their avian hosts during stressful and energy-demanding periods such as migration (Davidar and Morton, 1993; Valkiūnas, 2005; Garvin *et al.*, 2006).

In conclusion, we confirmed that active transmission of *Plasmodium relictum* (lineage SGS1) occurs in house martins in Europe. Additionally, we detected African and European MSP1 alleles in adult house martins,

suggesting two different areas of transmission for the *P. relictum* SGS1 lineage in this migratory bird species. These findings emphasize the importance of using multiple independent loci of avian *Plasmodium* parasites to understand transmission areas of blood parasites. Further studies exploring the transmission and species limit of avian malaria parasites are needed to evaluate the importance of migratory birds in spreading haemosporidian infections.

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REFERENCES

Beadell, J. S., Ishtiaq, F., Covas, R., Melo, M., Warren, B. H., Atkinson, C. T., Bensch, S., Graves, G. R., Jhala, Y. V, Peirce, M. A., Rahmani, A. R., Fonseca, D. M. and Fleischer, R. C. (2006). Global phylogeographic limits of Hawaii's avian malaria. *Proceedings. Biological sciences / The Royal Society* **273**, 2935–2944.

Bensch, S., Stjernman, M., Hasselquist, D., Ostman, O., Hansson, B., Westerdahl, H. and Pinheiro, R. T. (2000). Host specificity in avian blood parasites: a study of *Plasmodium* and *Haemoproteus* mitochondrial DNA amplified from birds. *Proceedings. Biological sciences / The Royal Society* **267**, 1583–1589.

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

Cramp, S. and Perrins, C. M. (1994). *Handbook of the Birds of Europe, the Middle East, and North Africa: The Birds of the Western Palearctic Volume VIII: Crows to Finches*.

Cosgrove, C. L., Wood, M. J., Day, MK. P. and Sheldon, B. C. (2008). Seasonal variation in Plasmodium prevalence in a population of blue tits *Cysnistes caeruleus*. *Journal of Animal Ecology* 77, 540–548.

Davidar, P. and Morton, E. S. (1993). Living with parasites: Prevalence of a blood parasite and its effect on survivorship in the Purple Martin. *Auk* **110**, 116–119.

Dunn, J. C., Goodman, S. J., Benton, T. G. and Hamer, K. C. (2014). Active blood parasite infection is not limited to the breeding season in a declining farmland bird. *The Journal of parasitology* **100**, 260–6.

Garvin, M. C., Szell, C. C. and Moore, F. R. (2006). Blood parasites of Nearctic-Neotropical migrant passerine birds during spring trans-Gulf migration: impact on host body condition. *The Journal of parasitology* **92**, 990–996.

Gerold, P., Schofieldb, L., Blackman, M. J., Holder, A. A. and Schwarz, R. T. (1996). Structural analysis of the glycosyl-phosphatidylinositol membrane anchor of the merozoite surface proteins-1 and -2 of *Plasmodium falciparum*. *Molecular and Biochemical Parasitology* **75**, 131–143.

Githeko, A. K., Lindsay, S. W., Confalonieri, U. E. and Patz, J. A. (2000). Climate change and vector-borne diseases: a regional analysis. *Bulletin of the World Health Organization* **78**, 1136–1147.

Hall, T. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41**, 95–98.

Hellgren, O., Waldenström, J., Peréz-Tris, J., Szöll Ösi, E., Hasselquist, D., Krizanauskiene, A., Ottosson, U. and Bensch, S. (2007). Detecting shifts of transmission areas in avian blood parasites - A phylogenetic approach. *Molecular Ecology* **16**, 1281–1290.

Hellgren, O., Kutzer, M., Bensch, S., Valkiunas, G. and Palinauskas, V. (2013). Identification and characterization of the merozoite surface protein 1 (msp1) gene in a host-generalist avian malaria parasite, *Plasmodium relictum* (lineages SGS1 and GRW4) with the use of blood transcriptome. *Malaria journal* **12**, 381.

Hellgren, O., Carter, A., Bensch, S., Albayrak, T., Dimitrov, D., Ewen, J., Kim, K. S., Lima, M., Lynn, M., Palinauskas, V., Ricklefs, R. E., Sehgal, R. N. M., Valkiūnas, G., Tsuda, Y. and Marzal, A. (2015) Global phylogeography of the avian malaria pathogen *Plasmodium relictum* based on MSP1 allelic diversity. *Ecography* Accepted.

Howe, L., Castro, I. C., Schoener, E. R., Hunter, S., Barraclough, R. K. and Alley, M. R. (2012). Malaria parasites (*Plasmodium* spp.) infecting introduced, native and endemic New Zealand birds. *Parasitology Research* **110**, 913–923.

Ilgunas, M., Palinauskas, V., Iezhova, T. A. and Valkiunas, G. (2013). Molecular and morphological characterization of two avian malaria parasites (Haemosporida: Plasmodiidae), with description of Plasmodium homonucleophilum n. Sp. *Zootaxa* **3666**, 49–61. doi:10.11646/zootaxa.3666.1.5.

Kazlauskiene, R., Bernotiene, R., Palinauskas, V., Iezhova, T. A. and Valkiunas, G. (2013). Plasmodium relictum (lineages pSGS1 and pGRW11): Complete synchronous sporogony in mosquitoes Culex pipiens pipiens. *Experimental Parasitology* **133**, 454–461. doi:10.1016/j.exppara.2013.01.008.

Lope, F. de. (1986). La biometría del avión común (*Delichon urbica urbica L.*) en Badajoz, España. *Ardeola* **33**, 171-176.

Lope, F. de. and Silva, E. da. (1998). La fidelidad al lugar de nidificación o de nacimiento en el avión común (*Delichon urbica urbica L.*) en Badajoz, España. *Ardeola* **3 9,**51-58

Lowe, S., Browne, M., Boudjelas, S. and De Poorter, M. (2000). 100 of the world's worst invasive alien species a selection from the global invasive species database. *The Invasive Species Specialist Group (ISSG) a specialist group of the Species Survival Commission (SSC) of the World Conservation Union (IUCN)* **12**, 12.

Marzal, A., Bensch, S., Reviriego, M., Balbontin, J. and De Lope, F. (2008). Effects of malaria double infection in birds: one plus one is not two. *Journal of evolutionary biology* **21**, 979–87.

Marzal, A., Ricklefs, R. E., Valkiūnas, G., Albayrak, T., Arriero, E., Bonneaud, C., Czirják, G. A., Ewen, J., Hellgren, O., Hořáková, D., Iezhova, T. A., Jensen, H., Križanauskienė, A., Lima, M. R., De Lope, F., Magnussen, E., Martin, L. B., Møller, A. P., Palinauskas, V., Pap, P. L., Pérez-Tris, J., Sehgal, R. N. M., Soler, M., Szöllősi, E., Westerdahl, H., Zetindjiev, P. and Bensch, S. (2011). Diversity, loss, and gain of malaria parasites in a globally invasive bird. *PLoS ONE* **6**, 8.

Marzal, A., Reviriego, M., Hermosell, I. G., Balbontín, J., Bensch, S., Relinque, C., Rodríguez, L., Garcia-Longoria, L. and de Lope, F. (2013a). Malaria infection and feather growth rate predict reproductive success in house martins. *Oecologia* **171**, 853–61.

Marzal, A., Asghar, M., Rodríguez, L., Reviriego, M., Hermosell, I. G., Balbontín, J., Garcia-Longoria, L., de Lope, F. and Bensch, S. (2013b). Co-infections by malaria parasites decrease feather growth but not feather quality in house martin. *Journal of Avian Biology* **44**, 437–444.

Marzal, A., García-Longoria, L., Cárdenas Callirgos, J. M. and Sehgal, R. N. (2015). Invasive avian malaria as an emerging parasitic disease in native birds of Peru. *Biological Invasions* **17**, 39-45.

Miller, L. H., Roberts, T., Shahabuddin, M. and McCutchan, T. F. (1993). Analysis

of sequence diversity in the *Plasmodium falciparum* merozoite surface protein-1 (MSP-1). *Molecular and Biochemical Parasitology* **59**, 1–14.

Møller, A. P. (2005). Parasitism and the regulation of host populations. *Parasitism and Ecosystems* **75**, 43–43.

Pajuelo, L., de Lope, F. and da Silva, E. (1992). Breeding biology of the House Martin (*Delichon urbica*) in Badajoz W Spain. *Ardeola* **39**, 15–23.

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

Piersma, T. and van der Velde, M. (2012). Dutch House Martins *Delichon urbicum* gain blood parasite infections over their lifetime, but do not seem to suffer. *Journal of Ornithology* **153**, 907–912.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (2002). *Molecular Cloning: A Laboratory Manual*. Cold Sprin.

Samuel, M. D., Hobbelen, P. H. F., Decastro, F., Ahumada, J. A., Lapointe, D. A., Atkinson, C. T., Woodworth, B. L., Hart, P. J. and Duffy, D. C. (2011). The dynamics, transmission, and population impacts of avian malaria in native Hawaiian birds: A modeling approach. *Ecological Applications* **21**, 2960–2973.

Schmid-Hempel, P. (2011). *Evolutionary parasitology: The integrated study of infections, immunology, ecology and genetics.*

Sol, D., Jovani, R. and Torres, J. (2003). Parasite mediated mortality and host immune response explain age-related differences in blood parasitism in birds. *Oecologia* **135**, 542–547.

Svensson, L., Grant, P. J. and Mullarney, K. (2009). *Collins bird guide*. HarperCollins, London.

Tumer, A. K. and Rose, C. (1989) *Swallows and Martins: an identification guide and handbook*. Taylor FJR (ed). Blackwell, Oxford.

Valkiūnas, G. (2005). *Avian malaria parasites and other Haemosporidia*. Boca Raton.

Valkiūnas, G., Zehtindjiev, P., Hellgren, O., Ilieva, M. and Iezhova T., B. S. (2007). Linkage between mitochondrial cytochrome b lineages and morphospecies of two avian malaria parasites , with a description of *Plasmodium* (Novyella) ashfordi sp . nov. *Parasitology Research* **100**, 1311–1322.

Van Riper III, C., Van Riper, S. G., Goff, M. L. and Laird, M. (1986). The epizootiology and ecological significance of malaria in Hawaiian land birds. *Ecological Monographs* **56**, 327–344. doi:10.2307/1942550.

Van Rooyen, J., Jenkins, T., Lahlah, N. and Christe, P. (2014). North-african house martins endure greater haemosporidian infection than their european counterparts. *Journal of Avian Biology*.

Waldenström, J., Bensch, S., Hasselquist, D. and Ostman, O. (2004). A new nested polymerase chain reaction method very efficient in detecting *Plasmodium* and *Haemoproteus* infections from avian blood. *The Journal of parasitology* **90**, 191–194.

Malaria infection affect negatively feather growth rate in house sparrows

I was taught that the way of progress was neither swift nor easy.

Marie Curie.

VI

Los parasites maláricos poseen un papel importante en la historia evolutiva de las aves ya que afectan a su éxito reproductivo, supervivencia y eficacia biológica. Recientemente, se ha mostrado que los parásitos maláricos pueden afectar a la velocidad de muda en especies migradoras. Sin embargo, el mecanismo entre la velocidad de muda, la salud del hospedador y la infección por parásitos maláricos permanece oculto. Ya que los recursos nutricionales y energéticos son limitados, en teoría se podría predecir que un individuo debe distribuir esta energía limitada entre los diferentes sistemas fisiológicos como el sistema inmunitario o el vuelo. En este capítulo, llevamos a cabo tres estudios diferentes en gorriones comunes para detectar la relación entre la velocidad de muda, la infección por malaria y la salud del hospedador. Primero, analizamos si los individuos infectados naturalmente y los no infectados diferían en su velocidad de muda. Después, examinamos si los gorriones infectados experimentalmente mudaban más lentamente que los no infectados. Y por último, evaluamos si un factor fisiológico como es la condición corporal (peso, hematocrito, grasa corporal, musculatura o la habilidad para volar verticalmente) estaba afectado por la muda o la infección experimental por malaria. Nuestros resultados muestran que la velocidad de muda se vio negativamente afectada por la infección parásita en condiciones naturales. Además, experimentalmente demostramos que la infección por *Plasmodium relictum* reduce dicha velocidad en gorriones comunes. Finalmente, demostramos un efecto negativo inducido por la muda y la infección por malaria en la grasa y la musculatura de los gorriones pero no en su peso ni en su capacidad para volar verticalmente. Estos resultados resaltan el papel del parásito de la malaria como un mecanismo potencial que puede afectar tanto a la velocidad de muda como a la eficacia biológica de su hospedador.

Palabras clave: Interacción parásito-hospedador; velocidad de muda; *Passer domesticus*; *Plasmodium*

Malaria infection affect negatively feather growth rate in the ubiquitous sparrow

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ABSTRACT

Malaria parasites have an important role in the evolutionary history of birds, as they have been shown to affect reproductive success, survival and fitness. Recently, it was shown that malaria parasites may have an effect on moult speed in a migratory species. However, little is known about the underlying mechanism between feather growth rate, host health conditions and malaria parasite infection. Because nutritional and energetic resources are limited, theory predicts that an individual must allocate the limited supply of resources between various physiological systems, such as immunity, flight and general condition. Here, we conducted three studies in the house sparrow in order to investigate whether a trade-off occurred among feather growth rate, malaria infection and host health conditions. First, we explored whether naturally infected and uninfected house sparrows differed in feather growth rate in the wild. Secondly, we examined this relationship by experimentally manipulating house sparrows in captivity. And finally, we evaluated whether a third physiological factor - individual condition (body mass, haematocrit, fat scores, muscle score and the ability of birds to perform hovering flights) - were affected by forced moult or malaria experimental infection. Our findings showed that the rectrix growth rate in house sparrows was negatively affected by haemosporidian infection in wild conditions. Moreover, we experimentally revealed that the infection with *Plasmodium relictum* reduced feather growth rate in house sparrows. To the best of our knowledge, this is the first study demonstrating the negative effects of malaria infection on the speed of moult of birds. Furthermore, our outcomes showed that malaria parasite also negatively affect the hematocrit of birds. Finally, we also revealed the negative effects of induced moult and malaria infection on fat and protein storage in house sparrows, but not on body mass and vertical hover capacity. These findings highlight the role of malaria parasites as a potential mechanism driving this trade-off to explain fitness differences in wild populations of birds.

Keywords: Host-parasite interactions; moult speed; *Passer domesticus*; *Plasmodium*.

INTRODUCTION

Feathers play a vital role in the life-history of birds. They are essential for flight, but they can also provide streamlining, insulation, camouflage, waterproofing and may act as sexual traits involved in female mate choice (Proctor and Lynch 1994; Kose et al. 1999; Kraaijeveld et al. 2004). Daily activities such as rubbing, preening and dust bathing all subject feathers to physical abrasion that causes wear in the plumage (Butler and Johnson 2004). Additionally, feathers are also exposed to factors such as ultraviolet light (Bergman 1982) and bacterial activity (Burtt and Ichida 1999) that lead to degradation. As damage accumulates, the functional properties of feathers are compromised, and hence birds must replace (moult) them to maintain plumage functions.

During moult, birds may suffer from increased vulnerability to predators (Lind 2001), a reduction in flight performance (Williams and Swaddle 2003), and/or a decrease in thermoregulatory capability (Ginn and Melville 1983). As such, it likely behooves birds to minimize moult duration (Hasselquist 1998) by maximizing feather growth rates (De La Hera et al. 2011). Evidence supporting this hypothesis comes from Gienapp and Merilä (2010) who showed that Siberian jays (*Perisoreus infaustus*) with faster feather growth rates had higher survival prospects. In addition, Marzal et al. (2013a) showed that house martins (*Delichon urbica*) with low feather growth rate during the preceding winter delayed egg-laying in the subsequent spring, leading to a decrease in clutch size and the number of reared chicks. Yet, while birds should ideally replace their old feathers regularly and rapidly for the reasons mentioned above, moult does require a significant investment of resources (Klaassen 1995; Murphy 1996), which could impose trade-offs with organs and functions (Murphy and Taruscio 1995; Murphy 1996; Nava et al. 2001). Because resources are usually limited in natural systems, increased investment in one physiological process leads to decreased investment in other processes (Roff 1992; Stearns 1992). As a consequence, the rate of feather growth during moult can be affected by factors such as body condition, nutritional status, physiological stress and disease (DesRochers et al. 2009; Moreno-Rueda 2010; Vágási et al. 2012). However, to the best of our knowledge, any study has analyzed how condition assessments (as body mass, hematocrit, fat scores and muscle score) or flight performance are affected by the moult process.

Avian malaria and related haemosporidian parasites (e.g. *Plasmodium* and *Haemoproteus*) are common parasites of many bird species (Valkiūnas 2005) and are known to provoke detrimental effects on their passerine hosts by decreasing host fitness (Marzal et al. 2005; Palinauskas et al. 2008; Valkiūnas 2005). *Plasmodium* species in particular are the most harmful and damage species of avian malaria, causing high rates of mortality (Van Riper III et al. 1986; Valkiūnas 2005), and affecting reproductive success (Merilä and Andersson 1999; Asghar et al. 2011), body mass (Marzal et al. 2008) and fitness (Lachish et al. 2011). During plumage replacement, malaria parasites may affect host resource allocation and thereby determine the growth rate of the feathers that are produced. Although some studies have shown that blood parasite infections are associated with the time of

moulting (Morales et al. 2007; Tarello 2007 also see Allander and Sundberg 1997), the effects of haemosporidian parasites on the growth rate of individual feathers are largely unknown. Also, malaria parasites infect hundreds of bird species worldwide, but a negative association between malaria infection and feather growth rate has been poorly investigated. Marzal et al. (2013a) found that rectrix growth rate in wintering house martins was negatively affected by blood parasite infection. They also found that birds co-infected with two haemosporidian lineages had the lowest inferred growth rate of their tail feathers as compared with uninfected and individuals infected with only a single lineage (Marzal et al. 2013b). Thus, it has been shown that malaria parasites may affect negatively feather growth rate under natural conditions (Marzal et al 2013a, 2013b). Because both processes, moult and infection, require significant amount of energy (Klaassen 1995; Murphy 1996; Valkiūnas 2005) limited natural resources might be distributed over these two processes. However, to date, any study has tested which of these events (moult or malaria infection) is more demanding in birds.

Here we conducted three studies in the house sparrow, *Passer domesticus*, one of the most ubiquitous hosts for avian malaria (Marzal et al. 2011). First we explored whether naturally infected and uninfected house sparrows differed in feather growth rate in the wild. If the effect of malaria infection on the growth rate of feathers seen in previous studies holds up in house sparrows under natural conditions, we would expect a negative relationship between malaria infection and feather growth rate in wild sparrows. Secondly, we examined this relationship by experimentally manipulating house sparrows in captivity, thereby removing many potentially confounding variables that are present in wild populations. If malaria parasites are the factor provoking a drop in feather growth rate, then we should expect a slower inferred growth rate of tail feathers in experimentally-infected compared to uninfected control birds while both groups are kept under standard captive conditions. Finally, in order to evaluate which event was more demanding (moult or infection by malaria parasites) we evaluated individual condition assessments (body mass, hematocrit, fat scores and muscle score) and the ability of birds to perform hovering flights (Veasey et al. 1998) after being experimentally-infected and/or forced to moult in the outermost tail feathers. We expected lower flight performance and a worse body condition in malaria experimentally-infected birds and those induced to moult compared to control birds. Moreover, we predicted negative trend in condition and flight performance from controls to moulting or experimentally-infected sparrows to individuals that received simultaneously both treatments (induced moult and malaria inoculation).

MATERIALS AND METHODS

Study site and sample collection

The three studies were conducted using all or a subset of a population of 100 adult house sparrows captured in January and February 2012 in mist-nets from the Universidad de Extremadura Badajoz campus (38°52'N, 6°58'W) in southwest Spain. At capture, we recorded body mass with a

Pesola spring balance to the nearest 0.1 g and collected a single microcapillary tube (70 µl) of blood from the brachial vein which was stored in 500 µl of SET buffer (0.15 M NaCl, 0.05 Tris, 0.001 M EDTA, pH 8.0) until DNA extraction. All birds were individually identified with numbered metal rings. Birds were then released in the aviaries of the Experimental Garden in the University of Extremadura. Molecular screening of blood samples collected at capture were used in determining naturally infected and uninfected individuals for study 1, a correlational study examining natural infection status versus feather growth rate. This initial molecular screening was also used to choose 10 *Plasmodium relictum* SGS1 (*p*SGS1) infected sparrows as donors and 38 uninfected individuals for study 2, where we examined infection status and burden versus feather growth rate, and study 3, where we evaluated the strength of trade-offs between infectious burden, body condition and feather growth. The rest of sparrows were released within three days of capture.

Moult induction and experimental infection

House sparrows were placed in aviaries in which each of several cages (3.5 x 1.5 x 2.5 m) contained a maximum of eight individuals. Birds were provided with water and food *ad libitum* and they stayed one month in the aviaries. Thirty-eight uninfected individuals were randomly assigned to one of four treatments: (1) moult group (N = 10), where sparrows were forced to moult their right outermost tail feathers by plucking them and were inoculated with 250 uL of the control solution, phosphate buffered saline (PBS) in the pectoral muscle; (2) infected group (N = 10), where sparrows were experimentally infected by intramuscularly inoculation of 250 uL of malaria infected blood mixture (100 uL blood from infected house sparrow donor, 25 uL 3.7 % sodium citrate, 125 uL 0.9% buffered saline) in the pectoral muscle (Palinauskas et al. 2008); (3) combined moult and infected group (N = 10), where sparrows received both malaria infection and forced moult treatments explained above; or (4) a control group (N = 8), where sparrows were inoculated with 250 uL of PBS.

Condition measurements and blood sampling

Immediately before the treatment and 4, 10 and 17 days post-treatment, all experimental individuals were weighed to the nearly 0.1 g, their pectoral muscle volume scored on a 0-2 ordinal scale (Gosler 1991), their visual fat scored on a 0-5 ordinal scale (Gosler 1996) and their blood collected. Blood was collected into two 70 uL samples heparinized microcapillary tubes from the brachial vein of each individual. One drop of one of these samples was used to create a blood smear for microscopic quantification of malaria parasites after treatment; the remainder of the sample was stored in 500 uL of SET buffer for molecular diagnosis of malaria infection (described below). The second microcapillary tube was centrifuged for 10 min at 11,000 r.p.m. to estimate haematocrit.

Vertical flight challenge

Vertical flight performance was quantified weekly by assessing the height of hovering flights in a Plexiglass™ vertical flight chamber (180 H x 21.5 W x 21.5 D cm) housed in a room free of other birds (Veasey et al. 1998; Altshuler et al. 2010).

All the trials were conducted in the same time of the day for all the individuals (between 0900 – 1030). The chamber included a trapdoor approximately 10 cm above the floor that was released to encourage flight when the trial began. To conduct each trial, a video camera was placed in the room prior to trials, equidistant from the flight chamber for all trials. A single bird was then captured from its cage in a separate room, placed in the flight box on the trapdoor, and given ~45 s to accommodate to the conditions. A 0.5 m section of nylon cord with 1.3 g weights every 10 cm was attached to each individual's left leg (Altshuler et al. 2010). Flight was induced by the sudden opening of trapdoor, which caused the sparrows to display their natural escape response, i.e. vertical flight, thereby lifting progressively more weight until reaching the maximum elevation. The summed height (cm) of all flights during a 30-second trial was used as score of performance.

Measuring feather growth rate

We plucked the right outermost tail feather from some individuals to determine feather grow rate of naturally infected and uninfected birds (for study 1) and to induce moulting of this feather (for studies 2 and 3). Once the right outermost tail feather was fully grown it was plucked again in order to compare feather grow rate between initial and renewal feathers in experimentally manipulated birds (studies 2 and 3). All feathers were stored in dry paper envelopes until laboratory analyses. Bird feathers have a series of light and dark bands perpendicular to the feather rachis. Each light and dark band taken together (one growth bar) represents 24 h of growth (Riddle 1908; Michener and Michener 1938; Grubb 2006). Thus, the number of dark bands indicates the number of days spent moulting these feathers. We measured the number of growth bars and the length of the right outermost rectrix feather in a Biorad XR gel documentation system in the laboratory following Shawkey et al. (2003). To visualize growth bars, we placed the feather in a light cabinet. We positioned a ruler (0,1 mm accuracy) near to the feather for use as a scale marker. Once contrast and resolution were optimized, a digital image of the feather was obtained. We measured the number of growth bars and the length of rectrix minus the calamus using ImageJ software (Abràmoff et al. 2004).

Molecular detection of blood parasite infections

Haemosporidian parasites (*Plasmodium* spp. and *Haemoproteus* spp.) were detected from blood samples using both microscopy (Valkiūnas 2005) and molecular methods (Waldenström et al. 2004). Blood smears were fixed in absolute methanol and stained with Giemsa the day of collection. The intensity of *p*SGS1 infection was quantified as number of parasites per 100 fields under 1,000X magnification with oil immersion (Godfrey et al. 1987). DNA from the avian blood samples was extracted using the standard phenol/chloroform/isoamyl alcohol method (Sambrook et al. 2002). Diluted genomic DNA (25 ng/uL) was used as a template in a polymerase chain reaction (PCR) assay for detection of the parasites using nested PCR-protocols described by Waldenström et al. (2004). Amplified products were evaluated by running 2.5 μL of the final PCR on a 2% agarose gel. All PCR experiments contained one negative control for every eight samples. The obtained sequences of 478 bp of the

cyt b were edited, aligned and compared in a sequence identity matrix using the program BioEdit (Hall 1999) to (1) determine naturally infected sparrows (study 1), and (2) to select *pSGS1*-infected sparrows as donors and uninfected sparrows for experimental manipulation (studies 2 and 3).

Statistical procedures

All analyses were run in IBM SPSS v22.0 and graphs made in GraphPad Prism v5.04. Blood samples from every malaria-exposed individual were found infected by d10 using molecular or microscopic techniques. Two experimental individuals infected by donor #5 (both in the infected group) had significantly higher burdens (>2 SD) than any other infected individuals; therefore these individuals were not included in analyses of burden. Initial visualization and analysis of the data did not suggest that any of the variables were significantly non-normal (based on regression residuals) nor did they support the use of cage, sex or donor as covariates in any analyses. We used a multivariate general linear model (GLM) to analyze the effect of burden and moult speed on body condition metrics (mass, hematocrit, fat score, muscle score and summed jumps in vertical flight performance) in the appropriate treatment groups. We analyzed paired data (Wilcoxon matched-pairs signed-ranks test) before and after treatment to test the effect that malaria infection and/or molt change health conditions of the host.

RESULTS

Study 1: Malaria infection and feather growth rate in wild conditions

We analysed 105 blood samples from wild house sparrows for malaria infection; 60 (57%) individuals were uninfected and 45 (43%) individuals were infected. There were differences in feather growth rate between infected and uninfected birds (N = 105; df = 1; F = 5.321; P = 0.023); infected house sparrows grew feathers more slowly than uninfected individuals [mean feather growth rate (SD): uninfected = 3.37 (0.26) mm / day; infected = 3.25 (0.26) mm / day] (Fig. 1).

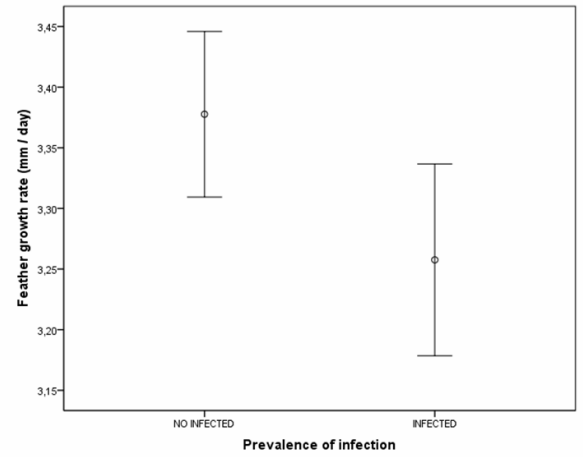

Figure 1. Feather growth rate (mm / day) for uninfected (N = 60) and infected house sparrows (N = 45). Error bars plots show means ± 95% of confidence interval.

Study 2: Malaria infection and feather growth rate in captivity

Only malaria infection explained significant variation in rectrix growth rate while controlling for other potentially influencing variables such as sex, muscle score, fat score, haematocrit and body mass (Table 1). Specifically, malaria-inoculated sparrows significantly decreased feather growth rate whereas uninfected control birds increased feather growth rate although not significantly (Mean variation in feather growth rate (SD): infected = - 0.50 (0.37) mm / day; control = 0.27 (0.33) mm / day; Estimate (SE) = 1.059 (0.294)). None of the interaction terms were statistically significant (P > 0.10) in all models (Table 1) (Figure 2).

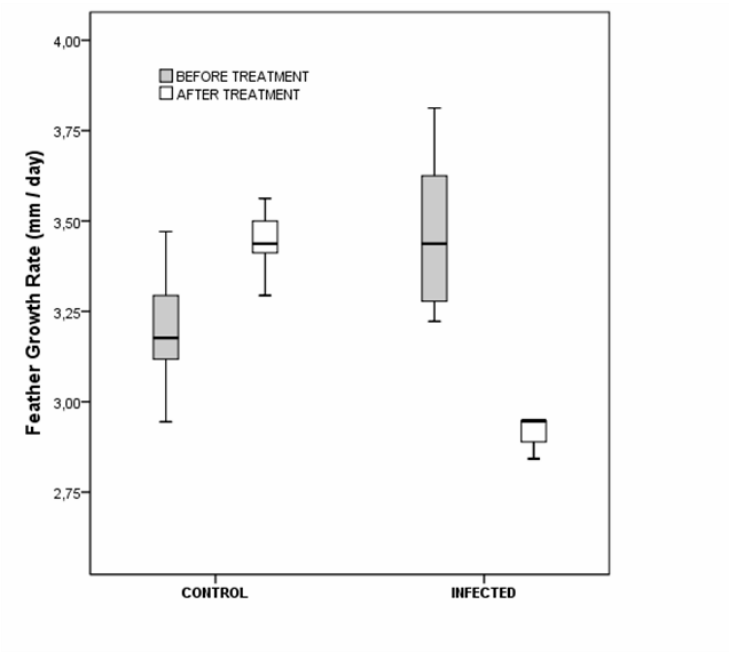

Figure 2. Feather growth rate for experimentally-infected (N = 10) and uninfected house sparrows (N = 10) before and after the forced moult.

Study 3: Malaria infection, feather growth rate, host condition and vertical flight performance

We found that all individuals increased their body mass during the experiment, independently of the treatments they received (Table 2). Body mass gain was marginally non-significant in individuals from control group (P = 0.09), while the increase in body mass was significant in birds from the rest of the treatments.

Table 2. Mean (SD) values for condition and performance of house sparrows before (1) and after (2) the experimental treatment. *P*-values in Wilcoxon signed-rank test less than 0.05 are marked in bold.

	Control				Moult				Infected				Moult-infected			
	Mean (SD) 1	*Mean (SD) 2*	Z	*P*	*Mean (SD) 1*	*Mean (SD) 2*	Z	*P*	*Mean (SD) 1*	*Mean (SD) 2*	Z	*P*	*Mean (SD) 1*	*Mean (SD) 2*	Z	*P*
Hematocrit	47.6 (6.90)	55.28 (2.51)	-2.03	**0.04**	46.4 (5.85)	55.90 (2.51)	-2.81	**0.00**	49.70 (4.62)	51.10 (3.87)	-0.92	0.35	51.80 (4.21)	53.11 (5.27)	-0.85	0.39
Fat	1.25 (0.46)	2.07 (0.73)	-2.06	**0.03**	2.00 (1.05)	2.75 (1.29)	-0.29	0.77	2.20 (0.91)	2.05 (0.89)	-0.29	0.77	1.30 (0.48)	1.60 (0.51)	-1.73	0.08
Muscle	0.50 (0.53)	1.07 (0.67)	-2.12	**0.03**	1.00 (0.47)	0.80 (0.58)	-1.24	0.21	0.60 (0.51)	0.70 (0.67)	-0.45	0.65	0.50 (0.52)	0.30 (0.48)	-0.82	0.41
Vertical flights (cm)	675.87 (325.24)	248.42 (252.83)	-2.19	**0.028**	736.10 (465.04)	777.00 (632.48)	-0.153	0.878	823.90 (559.70)	450.50 (402.55)	-2.293	**0.022**	831.40 (511.95)	893.11 (809.50)	-0.296	0.76
Body mass	25.61 (1.48)	26.17 (1.36)	-1.69	0.09	23.65 (1.64)	25.30 (1.25)	-2.65	**0.00**	25.50 (1.87)	26.70 (1.76)	-2.71	**0.00**	24.21 (1.49)	25.16 (1.22)	-2.24	**0.02**

Table 1. Results from the GLM explaining variation in the feather growth rate for individual house sparrows. Experimental infection, sex, body mass, haematocrit, fat score and muscle score were the predictor variables. Sample size was 20 individual.

Independent variable	Type III sum of squares	d.f.	F	P
Experimental infection	1.88	1	11.411	0.005
Sex	0.001	1	0.002	0.967
Body mass	0.001	1	0.009	0.927
Haematocrit	0.115	1	0.714	0.415
Fat score	0.060	1	0.372	0.553
Muscle score	0.004	1	0.022	0.883

Control and moult individuals significantly increased hematocrit after the treatment whereas hematocrit did not change in experimentally infected sparrows (both infected and moult-infected groups) (Table 2) (Figure 3).

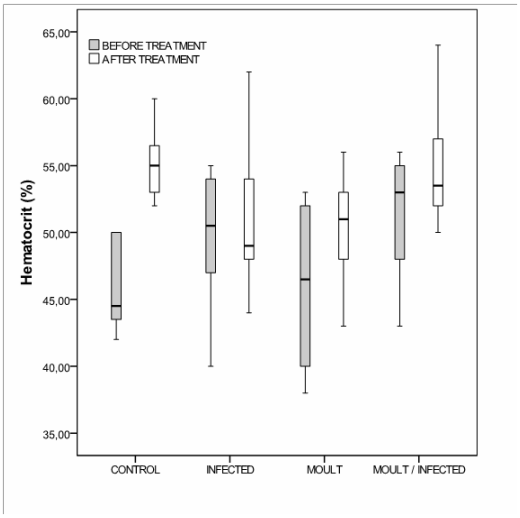

Figure 3. Hematocrit values among the four experimental groups (control, moult, infected and moult/infected), before and after the treatment. N = 40.

Neither fat score nor muscle score significantly changed in any moult and/or infected sparrows group during the experiment. Only individuals from control group significantly increased their muscle and fat scores after the treatment (Table 2).

Finally, both control and infected birds significantly decreased the summed height (cm) achieved during vertical flight performance (Table 2); this performance trait did not change in the other two groups.

DISCUSSION

Moult is a critical period within the annual cycle of birds because they suffer from reduced thermal insulation and flight performance (Williams and Swaddle 2003; Ginn and Melville 1983) and enhance the exposure to predators (Lind 2001). Since birds need to replace regularly their old feathers, natural selection should favour the regeneration of feathers as rapidly as possible. In migratory species it has been shown that stressors

such as blood parasites may reduce the feather growth rate during moult (Marzal et al. 2013b; Marzal et al. 2013a). However, as far as we know, this relationship has not been shown yet in resident species. Our findings showed that rectrix growth rate in resident wild house sparrows was negatively affected by haemosporidian infection. Moreover, we experimentally demonstrated that the infection with *Plasmodium relictum* SGS1 reduced feather growth rate in house sparrows. To the best of our knowledge, this is the first experimental study revealing the negative effects of malaria infection on the speed of moult of birds. These differences in feather growth rate between infected and non-infected house sparrows could be explained by consumption of host resources by the parasites. Host blood provides an ideal source of amino acids to the parasite, particularly during blood stage infection in which *Plasmodium* infects erythrocytes and asexually divides (Liu et al. 2006; Landfear 2011). The renewal of the feathers is a nutritionally and energetically costly process in birds requiring large amounts of amino acids, the main chemical compound of feathers (Murphy et al. 1990). Leucine, isoleucine and valine are essential amino acids to the completion of the complex life cycle of all *Plasmodium* species (Sherman 1977); they are also three of the four most abundant essential amino acids in bird feathers (Murphy et al. 1990). Consequently, the removal of essential amino acids from avian host by *Plasmodium* parasites could provoke this decrease in feather growth rate.

Alternatively, the lower growth rate in malaria-infected house sparrows can also be due to the need to mount an immune response against *Plasmodium* infection. Because both avian moult and mounting an immune response against parasite infection are physiologically demanding processes (Murphy 1996; Martin et al. 2003; Klasing 2004; Hasselquist and Nilsson 2012), a trade-off between these physiological activities is expected. In this sense, Martin (2005) showed feather growth was inversely related to cutaneous immune responses to phytohemagglutinin (PHA) in house sparrows. Moreover, Amat et al. (2007) found that regenerated feathers of immune-challenged greenfinches (*Carduelis chloris*) were more asymmetric than regenerated feathers from control birds. Also, Moreno-Rueda (2010) showed stimulation of the immune system with an antigen halved moult rate in house sparrows. The physiological mechanisms mediating feather renewal and response to malaria infection deserves further investigation.

Morphometric estimates of body condition typically reflect the size or nutrient reserves or the ability to resist parasites (Gosler 2004). Because a decrease in body mass is usually observed in haemosporidian infected (Valkiūnas 2005) and moulting birds (Portugal et al. 2007; Ndlovu et al. 2010), we expected a decrease in body mass during the experiment in moulting and / or infected house sparrows. But contrary to our expectations, sparrows increased body mass during the second experiment. These findings are in accordance with the 'environmental constraint' idea, suggesting that changes in body mass can be accounted for by local environmental conditions (Gehrold and Köhler 2013). Because both mounting and immune response and moulting requires substantial amount of nutrients (Martin et al. 2003;

Klasing 2004; Pap et al. 2008), the *ad libitum* food availability during the experiment might have masked effects of malaria infection and moulting on body mass. In support of this idea, it is known that environmental conditions such as food quantity or quality can significantly modify immune response in sparrows (Cornet and Sorci 2010). For example, Gonzalez et al. (1999) found that house sparrows provided with a high protein diet mounted stronger cell-mediated immune responses and had less detrimental effects of malaria compared to individuals experiencing a protein-poor diet.

Haematocrit has been widely used as an index of physiological condition in wild birds (see Fair et al. 2007 for a review), as it is often affected by food resources. For example, Sánchez-Guzmán et al. (2004) showed that haematocrit values in captive Northern Bald Ibis (*Geronticus eremita*) decreased as a response to poorer nutritional conditions. Moreover, hematocrit values were positively correlated with food availability in nestlings European serin (*Serinus serinus*) (Hoi-Leitner et al. 2001). Also, food supplementation increased haematocrit of fledgling Tengmalm`s owls (*Aegolius funereus*) (Santangeli et al. 2012). Similarly, our findings showed that moulting and control sparrows increased hematocrit, probably due to increased food availability during captivity. In contrast, hematocrit did not increase in malaria-infected house sparrows (infected and moult and infected groups), besides they were kept in the same captivity conditions as the rest of birds. These differences in hematocrit between infected and non-infected sparrows could be explained by the negative effects of blood parasite infection on hematological values. In this sense, it has been shown that an infectious disease such as malaria provokes anemia and decreased hematocrit values in birds (Campbell and Ellis 2007). Following this idea, Palinauskas et al. (2008) showed a significant decrease of hematocrit values in common crossbills (*Loxia curvirostra*) and siskins (*Spinus spinus*) experimentally infected with *Plasmodium relictum* SGS1. Therefore, the absence of increase of hematocrit values in infected house sparrows may be reflecting the red cell destruction and decreased red cell production provoked by malaria infection (Phillips and Pasvol 1992).

Under mild environmental conditions (e.g. water and food availability) energy intake exceeds expenditures, and thus a greater food intake is stored as large fat deposits under the skin, in the pectoral muscle, and in the abdominal cavity of birds (Davidson 1981; Dugan et al. 1981; Jenni-Eiermann et al. 2002). Because wild birds are usually under some nutritional stress, it is thus not surprising that captivity could lead to increases in fat and muscle scores. Consistent with this idea, Martin et al. (2012) recently showed that breast muscle size increased and fat score recovered after initial decline in house sparrows in captivity with *ad libitum* access to food and water. But these muscle and fat stores should be metabolized during increased rates of energy consumption. In this sense, moult may affect the metabolism by increased energy requirements and protein synthesis (Lindström et al. 1993). For example, plasma triglyceride levels decreased with body moult intensity in red knots (*Calidris canutus islandica*) (Jenni-Eiermann et al. 2002), and fat stores declined as moult progressed in tree

sparrows (*Passer montanus*) (Lind et al. 2004). Moreover, whole body protein turnover (synthesis and degradation) accelerated ~35% during moult in white-crowned sparrows (*Zonotrichia leucophrys*) (Murphy and Taruscio 1995; Taruscio and Murphy 1995). In addition, the production of an immune response and an infection may also raise the catabolism of proteins and lipids. For example, poultry species fighting infection often increase protein turnover and accelerate muscle protein breakdown to mobilize amino acids for proliferation of components of immune response (Butcher and Miles 2002). Also, fat scores declined significantly in Apapane (*Himatione sanguinea*) experimentally infected with malaria relative to uninfected controls (Yorinks and Atkinson 2000). Because renewal of feathers and fighting against malaria infection are both energetically demanding activities (Atkinson et al. 1988; Garvin et al. 2003; Martin et al. 2003; Pap et al. 2008), sparrows that were induced to moult and / or experimentally infected are expected to be more energetically constrained than control individuals. Our findings agree with this prediction, showing that neither experimentally-infected individuals (infected and moult/infected group) nor sparrows with induced moult (moult and moult/infected group) increased these measures of condition, probably revealing the energetic demands of feather renewal and immune response against malaria infection.

During moult birds may suffer a reduction in flight performance (Williams and Swaddle 2003). Hence, we expected sparrows which moult was forced (moult and moult + infected groups) to show a lower vertical flight performance than birds which moult was not induced. However, we found that both control and infected birds significantly decreased their vertical flight performance, but none of the sparrows which moult was forced (moult and moult + infected groups) varied their summed height achieved during vertical flight performance. It has been suggested that the study of the vertical flights through the flight box may entail habituation such that low values in week 0 followed by an increase and decline thereafter comprise both physical and mental changes (Martin et al. 2012). Thus, it might be that vertical flight performance variable could not be a suitable measurement to test the effect of moult and infection in long-term captured house sparrows.

To summarize, we demonstrated that malaria parasite negatively affect feather growth rate and hematocrit in a resident bird species. We also revealed the negative effects of induced moult and malaria infection on fat and protein storage in house sparrows, but not on body mass and vertical hover capacity. Future studies may go deeper in the relationship between infection by malaria parasite and amino acids concentration by comparing the amount of leucine, valine and isoleucine between infected and non-infected individuals. Additionally, as the body condition indexes we measured were unrelated to moult and malaria infection at the level of individuals, future studies may determine the mechanisms mediating molt-malaria infection trade-offs.

REFERENCES

Abràmoff MD, Magalhães PJ, Ram SJ (2004) Image Processing with ImageJ. Biophotonics Int 11:36–41.

Allander K, Sundberg J (1997) Temporal variation and reliability of blood parasite levels in captive yellowhammer Males *Emberiza citrinella*. J Avian Biol 28:325–330.

Altshuler DL, Dudley R, Heredia SM, McGuire JA (2010) Allometry of hummingbird lifting performance. J Exp Biol 213:725–734.

Amat JA, Aguilera E, Visser GH (2007) Energetic and developmental costs of mounting an immune response in greenfinches (*Carduelis chloris*). Ecol Res 22:282–287. d

Asghar M, Hasselquist D, Bensch S (2011) Are chronic avian haemosporidian infections costly in wild birds? J Avian Biol 42:530–537.

Atkinson CT, Forrester DJ, Greiner EC (1988) Pathogenicity of *Haemoproteus meleagridis* (Haemosporina: Haemoproteidae) in experimentally infected domestic turkeys. J Parasitol 74:228–239.

Bergman G (1982) Why are the wings of *Larus f. fuscus* so dark? Ornis Fenn 59:77–83.

Burtt EJ, Ichida J (1999) Occurrence of feather-degrading bacilli in the plumage of birds. Auk 116:364–372.

Butcher GD, Miles RD (2002) Interrelationship of Nutrition and Immunity.

Butler M, Johnson AS (2004) Are melanized feather barbs stronger? J Exp Biol 207:285–293.

Cornet S, Sorci G (2010) Parasite virulence when the infection reduces the host immune response. Proc Biol Sci 277:1929–1935.

Davidson N (1981) Survival of shorebirds (Charadrii) during severe weather: the role of nutritional reserves. Feed Surviv Srategies Estuar Org 15:231–249.

De La Hera I, Schaper SV., Díaz JA, et al. (2011) How much variation in the molt duration of Passerines can be explained by the growth rate of tail feathers? Auk 128:321–329.

DesRochers DW, Reed JM, Awerman J, et al. (2009) Exogenous and endogenous corticosterone alter feather quality. Comp Biochem Physiol - A Mol Integr Physiol 152:46–52.

Dugan P, Evans P, Goodyer L, Davidson N (1981) Winter fat reserves in shorebirds: disturbance of regulated levels by severe weather conditions. Ibis (Lond 1859) 123:359–363.

Fair J, Whitaker S, Pearson B (2007) Sources of variation in haematocrit in birds. Ibis (Lond 1859) 149:535–552.

Garvin MC, Homer BL, Greiner EC (2003) Pathogenicity of *Haemoproteus danilewskyi*, Kruse, 1890, in blue jays (*Cyanocitta cristata*). J Wildl Dis 39:161–169.

Gehrold A, Köhler P (2013) Wing-moulting waterbirds maintain body condition under good environmental conditions: a case study of Gadwalls (*Anas strepera*). J Ornithol 154:783–793.

Gienapp P, Merilä J (2010) Genetic and environmental effects on a condition-dependent trait: feather growth in Siberian jays. J Evol Biol 23:715–723.

Ginn HB, Melville D (1983) Moult in birds (BTO Guide 19). J Ornithol 116:263–280.

Godfrey RD, Fedynich AM, Pence DB (1987) Quantification of hematozoa in blood smears. J Wildl Dis 23:558–565.

Gonzalez G, Sorci G, Møller AP, et al. (1999) Immunocompetence and condition-dependent sexual advertisement in male house sparrows (*Passer domesticus*). J Anim Ecol 68:1225–1234.

Gosler AG (1991) On the use of greater covert moult and pectoral muscle as measures of condition in passerines with data for the Great Tit *Parus major*. Bird Study 38:1–9.

Gosler AG (1996) Environmental and social determinants of winter fat storage in the great tit *Parus major*. J Anim Ecol 65:1–17.

Grubb JTC (2006) Ptilochronology: feather time and the biology of birds. Book. doi: 10.1650/0010-5422(2003)105[0392:BR]2.0.CO;2

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

Hasselquist D (1998) Polygyny in great reed warblers: a long-term study of factors contributing to male fitness. Ecology 79:2376–2390.

Hasselquist D, Nilsson JÅ (2012) Physiological mechanisms mediating costs of immune responses: What can we learn from studies of birds? Anim Behav 83:1303–1312.

Hoi-Leitner M, Romero-Pujante M, Hoi H, Pavlova A (2001) Food availability and immune capacity in serin (*Serinus serinus*) nestlings. Behav Ecol Sociobiol 49:333–339.

Jenni-Eiermann S, Jenni L, Piersma T (2002) Plasma metabolites reflect seasonally changing metabolic processes in a long-distance migrant shorebird (*Calidris canutus*). Zoology (Jena) 105:239–246.

Klaassen M (1995) Moult and basal metabolic costs in males of two subspecies of stonechats: the European *Saxicola torquata rubicula* and the East African *S. t. axillaris*. Oecologia 104:424–432.

Klasing KC (2004) The costs of immunity. Acta Zool Sin 50:961–969.

Kose M, Mand R, Møller AP (1999) Sexual selection for white spots in the barn swallow in relation to habitat choice in feather lice. Anim Behav 15:380–389.

Kraaijeveld K, Gregurke J, Hall C, Komdeur J, Mulder RA (2004). Mutual ornamentation, sexual selection and social dominance in the black swan. Behave Ecol 15:424–43

Lachish S, Knowles SCL, Alves R, et al. (2011) Fitness effects of endemic malaria infections in a wild bird population: the importance of ecological structure. J Anim Ecol 80:1196–206.

Landfear SM (2011) Nutrient transport and pathogenesis in selected parasitic protozoa. Eukaryot Cell 10:483–493.

Lind J (2001) Escape flight in moulting Tree Sparrows (*Passer montanus*). Funct Ecol 15:29–35.

Lind J, Gustin M, Sorace A (2004) Compensatory bodily changes during moult in Tree Sparrows *Passer montanus* in Italy. Ornis Fenn 81:75–83.

Lindström A, Visser GH, Daan S (1993) The energetic cost of feather synthesis is proportional to basal metabolic rate. Physiol Zool 66:490–510.

Liu J, Istvan ES, Gluzman IY, et al. (2006) *Plasmodium falciparum* ensures its amino acid supply with multiple acquisition pathways and redundant proteolytic enzyme systems. Proc Natl Acad Sci U S A 103:8840–8845.

Martin LB (2005) Trade-offs between molt and immune activity in two populations of house sparrows (*Passer domesticus*). Can J Zool 83:780–787.

Martin LB, Brace A, Urban A, et al. (2012) Does immune suppression during stress occur to promote physical performance? J Exp Biol.

Martin LB, Scheuerlein A, Wikelski M (2003) Immune activity elevates energy expenditure of house sparrows: a link between direct and indirect costs? Proc Biol Sci 270:153–158.

Marzal A, Asghar M, Rodríguez L, et al. (2013a) Co-infections by malaria parasites decrease feather growth but not feather quality in house martin. J Avian Biol 44:437–444.

Marzal A, Bensch S, Reviriego M, et al. (2008) Effects of malaria double infection in birds: one plus one is not two. J Evol Biol 21:979–87.

Marzal A, de Lope F, Navarro C, Møller AP (2005) Malarial parasites decrease reproductive success: an experimental study in a passerine bird. Oecologia 142:541–5.

Marzal A, Reviriego M, Hermosell IG, et al. (2013b) Malaria infection and feather growth rate predict reproductive success in house martins. Oecologia 171:853–61.

Marzal A, Ricklefs RE, Valkiūnas G, et al. (2011) Diversity, Loss, and Gain of Malaria

Parasites in a Globally Invasive Bird. PLoS One 6:8.

Merilä J, Andersson M (1999) Reproductive effort and success are related to haematozoan infections in blue tits. Ecoscience 6:421–428.

Michener H, Michener JR (1938) Bars in flight feathers. Condor 40:149–160.

Morales J, Moreno J, Merino S, et al. (2007) Early moult improves local survival and reduces reproductive output in female pied flycatchers. Ecoscience 14:31–39.

Moreno-Rueda G (2010) Uropygial gland size correlates with feather holes, body condition and wingbar size in the house sparrow *Passer domesticus*. J Avian Biol 41:229–236.

Murphy ME (1996) Energetics and nutrition of molt. Avian Energ. Nutr. Ecol. pp 158–198.

Murphy ME, King JR, Taruscio TG, Geupel GR (1990) Amino-acid composition of feather barbs and rachises in 3 species of *Pygoscelid* penguins - Nutritional implications. Condor 92:913–921.

Murphy ME, Taruscio TG (1995) Sparrows increase their rates of tissue and whole-body protein synthesis during the annual molt. Comp Biochem Physiol - A Physiol 111:385–396.

Nava MP, Veiga JP, Puerta M (2001) White blood cell counts in house sparrows (*Passer domesticus*) before and after moult and after testosterone treatment. Can J Zool-Rev Can Zool 79:145–148.

Ndlovu M, Cumming GS, Hockey PAR, Leo WB (2010) Phenotypic flexibility of a southern African duck *Alopochen aegyptiaca* during moult: do northern hemisphere paradigms apply? J Avian Biol 41:558–564.

Palinauskas V, Valkiūnas G, Bolshakov CV, Bensch S (2008) *Plasmodium relictum* (lineage P-SGS1): effects on experimentally infected passerine birds. Exp Parasitol 120:372–380.

Pap PL, Vágási CI, Czirják GÁ, Barta Z (2008) Diet quality affects postnuptial molting and feather quality of the house sparrow (*Passer domesticus*): interaction with humoral immune function? Can J Zool 86:834–842.

Phillips RE, Pasvol G (1992) Anaemia of *Plasmodium falciparum* malaria. Baillieres Clin Haematol 5:315–30.

Portugal SJ, Green JA, Butler PJ (2007) Annual changes in body mass and resting metabolism in captive barnacle geese (*Branta leucopsis*): the importance of wing moult. J Exp Biol 210:1391–1397.

Proctor N, Lynch P (1994) Manual of Ornithology : Avian Structure and Function. Am Sci 82:288–289.

Riddle O (1908) The genesis of fault-bars in feathers and the cause of alternation of light and dark fundamental bars. Biol Bull 14:328 – 370.

Roff DA (1992) The evolution of life-histories. Nat Methods.

Sambrook J, Fritsch EF, Maniatis T (2002) Molecular Cloning: A Laboratory Manual, Cold Sprin. Cold Spring Harb Lab 3:2344.

Sánchez-Guzmán JM, Villegas A, Corbacho C, et al. (2004) Response of the haematocrit to body condition changes in Northern Bald Ibis *Geronticus eremita*. Comp Biochem Physiol - A Mol Integr Physiol 139:41–47.

Santangeli A, Hakkarainen H, Laaksonen T, Korpimäki E (2012) Home range size is determined by habitat composition but feeding rate by food availability in male Tengmalm's owls. Anim Behav 83:1115–1123.

Shawkey MD, Beck ML, Hill GE (2003) Use of a gel documentation system to measure feather growth bars. J F Ornithol 74:125–128.

Sherman IW (1977) Amino acid metabolism and protein synthesis in malarial parasites. Bull World Health Organ 55:265–276.

Stearns SC (1992) The evolution of life histories. Divers Distrib.

Tarello W (2007) Clinical signs and response to primaquine in falcons with *Haemoproteus tinnunculi* infection. Vet Rec 161:204–205.

Taruscio TG, Murphy ME (1995) 3-Methylhistidine excretion by molting and non-molting sparrows. Comp Biochem Physiol - A Physiol 111:397–403.

Vágási CI, Pap PL, Vincze O, et al. (2012) Haste makes waste but condition matters: Molt rate-feather quality trade-off in a sedentary songbird. PLoS One 7:7.

Valkiūnas G (2005) Avian malaria parasites and other Haemosporidia, Boca Raton.

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

Veasey J, Metcalfe N, Houston D (1998) A reassessment of the effect of body mass upon flight speed and predation risk in birds. Anim Behav 56:883–889.

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

Williams EV, Swaddle JP (2003) Moult, flight performance and wingbeat kinematics during take-off in European starlings *Sturnus vulgaris*. J Avian Biol 34:371–378.

Yorinks N, Atkinson CT (2000) Effects of malaria on activity budgets of experimentally infected juvenile apapane (*Himatione sanguinea*). Auk 117:731–378.

CONCLUSIONS

1. Birds infected with *Haemoproteus* and *Leucocytozoon* exhibit a more intense escape behavior (fear screams and struggling) when simulated a predator attack than non-infected birds.

2. Bird species exposed to a greater diversity of habitats (generalist) have higher prevalence of blood parasites than specialist bird species (exposed to a lower diversity of habitats).

3. Avian malaria parasite (*Plasmodium* spp.) may manipulate the escape behavior of their avian hosts, as the decrease in the intensity of escape behaviour in house sparrows after anti-malaria medication has shown.

4. For the first time, avian malaria parasite *Plasmodium relictum* SGS1 has been detected infecting Neotropical birds in the Americas, thus representing a serious menace for bird conservation.

5. Chitinase gene has been identified, for the first time, in one of the most prevalent avian malaria parasites *Plasmodium relictum.*

6. The detection of the two copies (short and long) in two mitochondrial lineages of *P. relictum* (GRW4 and SGS1) sheds light on the phylogenetic relationship of the chitinase gene in the genus *Plasmodium*.

7. Because of the high variability detect in the chitinase gene, it could be used to study the genetic population structure in isolates from different host species and geographic regions.

8. *Plasmodium relictum* SGS1 lineage may be actively transmitted in Europe in Afro-Paleartic migratory bird species, as the detection of juvenile house martins infected with this malaria lineage has shown.

9. All the juveniles and most of adult house martins were infected with a European transmitted malaria MSP1 allele, whereas the less number of adult birds were infected with two African transmitted MSP1 alleles.

10. The infection with avian malaria (*Plasmodium relictum*) decreases the feather growth rate in house sparrows.

11. This blood parasite infection also provokes negative effects on haematocrit and fat and protein storages in house sparrows.

Supplementary material

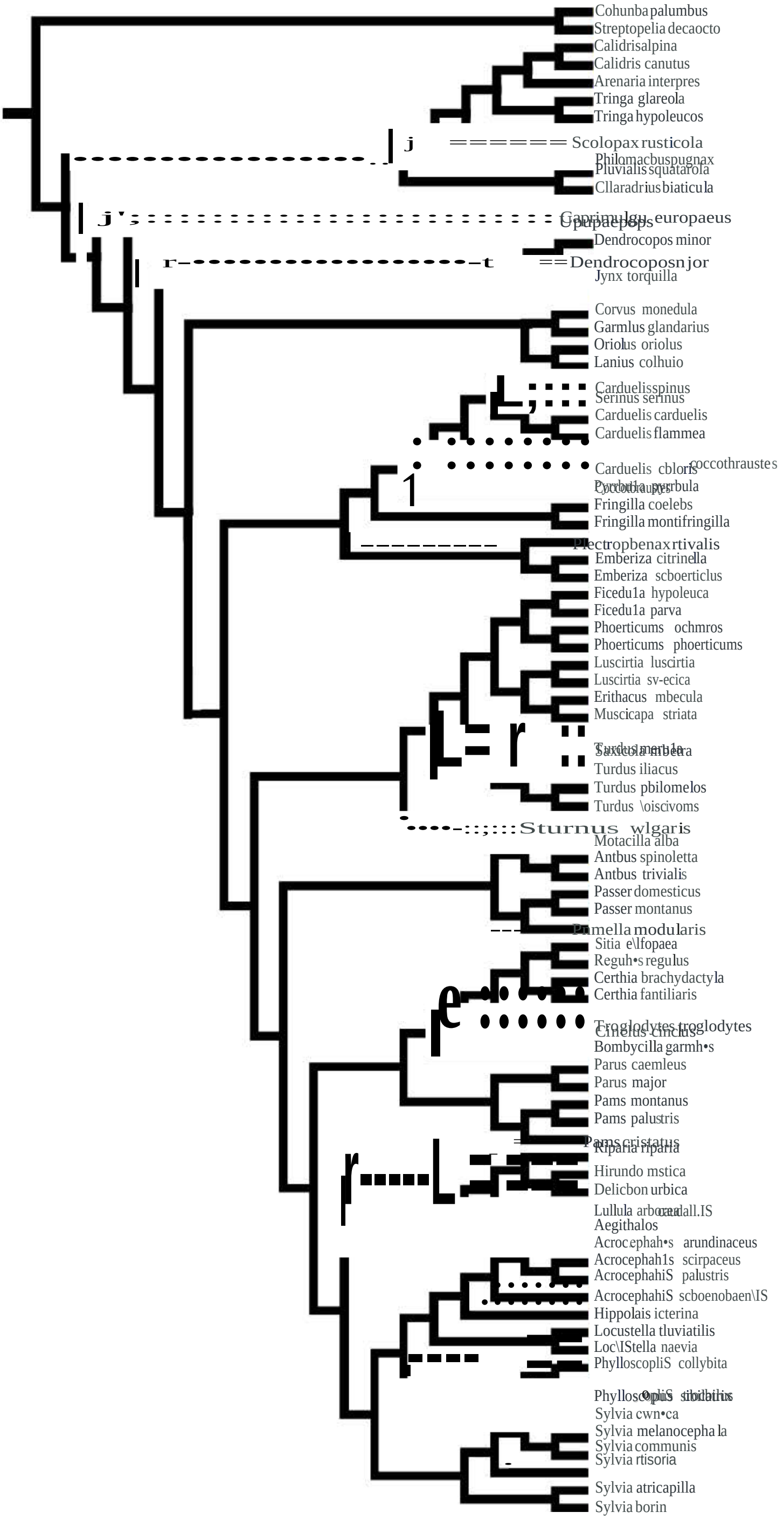

Figure Al. Phylogenetic relationship between the 85 species included in the study on parasite prevalence and escape behavior.

Table A1. Innovation behaviour (feeding innovations and FID (Flight Initiation Distance)), habitat complexity, habitat generalism (number of habitats), prevalence of blood parasite, escape behaviour, sample size, mass, migration and coloniality for all the avian host analyzed in this study.

Species	log Feeding innovations	log Research effort	log FID	Habitat Complex	No habitats	*Haemoproteus*	*Plasmodium*	*Leucocytozoon*	Biting	Fear scream	Feather loss	Tonic immobility (s)	Alarm	Wriggle	N Capture behaviour	Mass (g)	Migration	Coloniality
Acrocephalus arundinaceus	0	2.537	0	0	4	0.220	0.183	0.061	0	0	0	30	0	0	1	30.350	1	0
Acrocephalus palustris	0	0	0.946	0	14	0.530	0	0.132	0	0.330	0	13.670	0	1.330	3	12	1	0
Acrocephalus schoenobaenus	0	2.444	0.879	0	7	0.283	0.202	0.116	0	0	0	13	0	0.800	5	11.900	1	0
Acrocephalus scirpaceus	0	0	0.828	0	10	0.221	0.117	0.102	1	0	0	30	0	1	1	11.800	1	0
Aegithalos caudatus	0.778	2.223	0.701	2	11	0	0	0.127	0	0	0	1	0	0.330	3	8.800	0	0
Anthus spinoletta	0.301	2.288	0.619	0	16	0	0	0.250	0	0	0	21	0.250	1.500	4	21.450	1	0
Anthus trivialis	0	0	1.008	1	9	0.272	0	0.272	0.070	0.030	0.070	22.450	0.240	0.790	29	23.400	1	0
Arenaria interpres	0.903	2.483	1.312	0	5	0	0	0	0	0	0	0	0.500	0.500	2	107.500	1	0
Bombycilla garrulus	0.699	2.207	0	2	5	0	0	0.281	1	0	0	0	1	2	1	50	1	0
Calidris alpina	0	2.745	0	0	5	0	0	0	0	0	0	15.730	0.200	0.730	30	43.050	1	0
Calidris canutus	0.301	2.580	1.415	0	1	0	0	0	0	0.390	0	9.230	0.190	2.130	31	137	1	0
Caprimulgus europaeus	0	0	0	3	15	0.314	0.220	0.220	0	1	1	3	0	1	1	85	1	0
Carduelis carduelis	0.477	2.373	0.883	2	11	0.358	0.164	0	0.350	0.050	0.160	17.840	0.920	0.540	37	15.600	1	0
Carduelis chloris	0.845	2.465	0.819	2	9	0.416	0.276	0	0.530	0.100	0.230	16.780	0.690	0.960	81	27.650	1	0
Carduelis flammea	0.477	2.188	0.653	1	14	0.188	0	0.284	0.330	0	0.270	10.900	0.470	0.830	30	13.050	1	0
Carduelis spinus	0.778	2.384	0.684	2	3	0.124	0.307	0.243	0.240	0.180	0.150	8.060	0.240	1.030	34	13.800	1	0
Certhia brachydactyla	0	0	0.850	2	5	0	0	0	0	0	1	30	1	0	1	9.150	0	0
Certhia familiaris	0.301	2.354	0.650	2	4	0.194	0	0.137	0.100	0.600	0.050	8.800	0.150	1.050	20	9.150	0	0
Charadrius hiaticula	0	2.413	1.293	0	7	0	0	0	0	0	0	9.250	0.600	0.600	5	63.250	1	0
Cinclus cinclus	0.602	2.663	0	0	5	0	0	0.464	0.200	0.600	0.200	0	1	3	5	61.900	1	0
Coccothraustes coccothraustes	0.477	2.083	0.934	2	13	0.134	0.271	0.333	1	0.524	0.095	19.570	0.050	1.190	21	54.700	1	0

Columba palumbus	0.301	2.442	1.294	2	10	0.524	0.385	0.372	0	0	1	0.500	0	1.500	2	494.500	1	0
Corvus monedula	0.845	2.640	1.426	2	2	0	0.064	0.064	1	0.333	0	3.670	0	0.667	6	249	1	1
Delichon urbica	0.845	2.844	0.802	0	4	0.342	0	0	0.180	0.270	0	2.270	0.270	0.730	11	19.550	1	1
Dendrocopos major	1.041	2.332	1.128	2	5	0	0	0	1	0.600	0	0.750	0	2.200	5	89.650	0	0
Dendrocopos minor	0.301	1.778	0	2	3	0	0	0	0	0	0.250	30	0	0.500	4	25.500	0	0
Emberiza citrinella	0	0	1	1	10	0.461	0.320	0.183	0.080	0	0.140	8.750	0.920	0.810	36	26.750	0	0
Emberiza schoeniclus	0.301	2.303	0.992	0	15	0.075	0	0.295	0.730	0	0.190	7.290	0.390	1.520	31	18.800	1	0
Erithacus rubecula	0.845	2.712	0.710	2	3	0.249	0.092	0.196	0.100	0.060	0.070	11.520	0.100	0.930	71	16.350	1	0
Ficedula hypoleuca	0.477	2.997	0.732	2	10	0.407	0.197	0.127	0.030	0.170	0.030	6.310	0.090	1.600	35	14.350	1	0
Ficedula parva	0	2.111	0	2	3	0	0	0	0	0.500	0	5.500	0	0	2	11.700	1	0
Fringilla coelebs	0.845	2.763	0.854	2	10	0.561	0.120	0.388	0.850	0.170	0.150	12.980	0.300	1.060	124	24.200	1	0
Fringilla montifringilla	0.301	2.418	0	2	3	0.426	0.100	0.707	0.910	0.120	0.210	5.210	0.740	1.380	34	22.650	1	0
Garrulus glandarius	1	2.330	1.070	2	7	0.248	0.191	1.086	1	0.143	0.714	9.570	0	1.286	7	161.700	0	0
Hippolais icterina	0	0	0.876	2	10	0.558	0.287	0	0.470	0.890	0.080	6.580	0.060	2.220	36	13.300	1	0
Hirundo rustica	1.146	3.042	1.007	0	4	0.083	0.044	0.083	0.070	0.013	0.002	9.470	0.120	0.720	870	19.100	1	1
Jynx torquilla	0	2.196	0	2	5	0.257	0	0.367	0.200	0.200	0	3.200	0	1.800	5	37.350	1	0
Lanius collurio	0.602	2.651	0.862	1	16	0.604	0.219	0.126	1	0.220	0	20.220	0	1.040	27	30.700	1	0
Locustella fluviatilis	0	0	0	0	10	0	0	0	0	0	0	0	0	1	1	18.800	1	0
Locustella naevia	0	0	1.177	0	11	0	0	0	0	0.290	0.140	6.570	0	0.710	7	12.700	1	0
Lullula arborea	0	0	1.084	0	11	0.524	0	0	0	0	0	30	0	0	2	30.050	1	0
Luscinia luscinia	0	0	1.201	1	5	0.791	0.172	0.133	0.040	0.080	0.211	21.250	0	0.632	19	25	1	0
Luscinia svecica	0.301	2.365	0	1	7	0	0	0.421	0	0	0.360	1.270	0.080	2	11	18.250	1	0
Motacilla alba	0.954	2.571	1.054	0	11	0.245	0.140	0.657	0.530	0.030	0.030	9.420	0.660	1.420	38	20.750	1	0
Muscicapa striata	0.903	2.286	0.929	2	13	0.432	0.133	0.421	0.320	0.490	0.120	14.290	0.150	1.710	41	15.500	1	0
Oriolus oriolus	0.477	2.155	1.598	2	8	0.625	0.186	0.161	1	1	1	30	0	1	1	68.500	1	0

Parus caeruleus	1.041	2.932	0.744	2	9	0.174	0.091	0.335	0.020	0.520	0.080	8.100	0.560	1.120	50	11.750	0	0
Parus cristatus	0	2.196	0.801	2	3	0	0	0	0.250	0.500	0.250	23	0	1	4	11.150	0	0
Parus major	1.176	3.221	0.718	2	8	0.266	0.169	0.263	0.950	0.230	0.120	11.120	0.300	1.220	74	18.500	0	0
Parus montanus	0	2.544	0	2	11	0.195	0.259	0.482	1	0.500	0	30	0	1.500	2	11.650	0	0
Parus palustris	0.602	2.382	0.747	2	7	0.184	0	0	0.714	0	0	2.860	0	0.880	26	11.900	0	0
Passer domesticus	1.279	3.211	0.583	0	6	0.178	0.301	0.065	0.690	0.040	0	5.850	0.380	1.080	48	30.350	0	1
Passer montanus	0.602	2.671	0.708	1	4	0.331	0.353	0	0.270	0	0.020	14.960	0.100	1.080	48	21.700	0	1
Philomachus pugnax	0.477	2.491	0	0	5	0	0	0	0	0	0.040	5.330	0	1.750	24	140.500	1	0
Phoenicurus ochruros	0	2.480	0.842	0	3	0	0	0	0.120	0.060	0.120	18.940	0.240	1.060	17	16.100	1	0
Phoenicurus phoenicurus	0	2.358	0.959	2	7	0.217	0.132	0.382	0.190	0.150	0.070	15.970	0.450	1.433	67	15.900	1	0
Phylloscopus collybita	0.903	2.545	0.812	2	9	0.194	0	0.221	0.060	0.140	0.220	9.940	0.190	0.750	36	7.700	1	0
Phylloscopus sibilatrix	0	0	0	2	6	0.213	0	0	0.300	0.350	0	22.910	0.090	1.090	23	9.100	1	0
Phylloscopus trochilus	0.301	2.622	0.798	2	13	0.333	0.109	0.208	0.080	0.060	0	12.010	0.140	1.080	79	9.350	1	0
Plectrophenax nivalis	0	2.267	0	0	5	0	0	0	1	0	0	30	0	1	1	37.350	1	0
Pluvialis squatarola	0	2.310	1.708	0	7	0	0	0.340	0	0	0	3.250	0.500	0.500	4	227	1	0
Prunella modularis	0.699	2.318	0.706	2	18	0.242	0.054	0.236	0	0.030	0.280	6.690	0.130	1.220	32	18.950	1	0
Pyrrhula pyrrhula	0.699	2.301	0.779	2	10	0.183	0	0.248	0.170	0.250	0.130	9.250	0.500	1.420	24	31.050	1	0
Regulus regulus	0.699	2.279	0.652	2	4	0	0	0.150	0	0.200	0.030	9.660	0.550	0.670	30	5.800	1	0
Riparia riparia	0.778	2.731	1.364	0	7	0	0	0.104	0	0	0	1	0	0.500	2	13.150	1	1
Saxicola rubetra	0	2.212	1.221	0	10	0.441	0	0.279	0	0.400	0.200	16.200	0	1.600	5	16.600	1	0
Scolopax rusticola	0.301	2.487	1.041	2	8	0.350	0	0.245	1	0	1	16	0.500	1	2	309.500	1	0
Serinus serinus	0	0	0.766	2	6	0.348	0.217	0	0.090	0	0.450	18.500	0.270	0.910	11	11.950	1	0
Sitta europaea	0.477	2.441	0.819	2	8	0.370	0.210	0.210	0.667	0	0	1.670	0	1	3	23.900	0	0
Streptopelia decaocto	0.477	2.619	0.731	2	4	0	0	0	0	0	0.800	15.500	0	1.200	5	201.500	0	0
Sturnus vulgaris	1.146	3.281	0.989	2	8	0.034	0.109	0.034	0.560	0.500	0.090	5.790	0.940	2	34	80.500	1	1

Sylvia atricapilla	1	2.715	0.766	2	7	0.536	0.075	0.177	0.270	0.150	0.110	14.560	0.020	1.300	66	18.850	1	1
Sylvia borin	0	0	0.820	2	9	0.457	0.103	0.216	0.380	0.500	0.240	13.460	0	1.700	50	19.050	1	1
Sylvia communis	0.477	2.272	0.911	1	12	0.535	0.133	0.196	0.070	0.170	0.240	15.830	0.050	1.020	42	14.500	1	1
Sylvia curruca	0	2.190	0.723	1	15	0.316	0.188	0.133	0	0.110	0	14.450	0.032	0.680	62	12.400	1	1
Sylvia melanocephala	0	0	0.822	1	7	0	0	0	0	0	1	16	0	2	2	13.450	1	1
Sylvia nisoria	0	1.996	0	1	11	0.719	0	0	0.190	0.190	0.060	17.070	0	1.560	16	24.350	1	1
Tringa glareola	0	0	0	0	5	0.388	0	0	0	0	0	2.500	0.330	1.670	3	67.500	1	1
Tringa hypoleucos	0	0	1.230	0	9	0	0	0.187	0	0	0	30	0	2	1	47.750	1	1
Troglodytes troglodytes	0.301	2.515	0.751	2	14	0.091	0	0.091	0.140	0.060	0.060	2.580	0.390	1.640	36	8.900	1	1
Turdus iliacus	0.602	2.318	1.106	2	5	0.393	0.598	0.357	0.778	0	0.556	11.440	0.778	1.222	9	62.850	1	1
Turdus merula	1.477	2.936	0.852	2	11	0.223	0.333	0.290	0.640	0.300	0.810	18.900	0.460	1.340	164	95.850	1	1
Turdus philomelos	1.079	2.540	0.888	2	7	0.372	0.198	0.586	0.970	0.480	0.640	15.390	0.390	1.480	66	70.500	1	1
Turdus viscivorus	0.699	2.041	1.286	2	7	0.194	0	0.395	0.833	0.500	1	6.170	0	1.330	6	117.800	1	1
Upupa epops	0	0	1.300	0	12	0.140	0.140	0.140	0	0	0	10	0	0.330	3	67.050	1	1

Table A2. Infection by *Haemoproteus*, *Leucocytozoon* and *Plasmodium* in relation to escape behaviour and frequency of feeding innovations. Test statistics refer to linear estimated and their standard errors (SE) and the associated *P*-values in phylogenetic analyses weighted by sample size. Effect sizes (r) are showed with its 95% CIs.

					95% CIs		
Factor	Value	SE	*t*	Effect size	Lower	Upper	*P*
Haemoproteus							
log Research effort	-0.051	0.093	-0.55	-0.064	-0.285	0.164	0.579
Alarm call	-0.005	0.090	-0.06	-0.007	-0.232	0.219	0.952
Biting	0.104	0.061	1.68	0.191	-0.035	0.399	0.095
Wriggle	0.011	0.045	0.25	0.029	-0.197	0.252	0.803
Feather loss	0.105	0.082	1.29	0.148	-0.080	0.361	0.201
Fear scream	0.137	0.099	1.37	0.157	-0.071	0.370	0.172
log Tonic Immobility (s)	0.006	0.124	0.04	0.004	-0.221	0.230	0.961
log Mass (g)	-0.290	0.095	-3.06	-0.335	-0.521	-0.118	0.003
λ = 0.000, residual SE=0.067, df=76							
Leucocytozoon							
log Research effort	-0.016	0.072	-0.22	-0.025	-0.201	0.117	0.819
Alarm call	0.037	0.085	0.43	0.049	-0.177	0.272	0.662
Biting	0.185	0.058	3.17	0.345	0.130	0.529	0.002
Wriggle	0.029	0.042	0.67	0.077	-0.150	0.297	0.499
Feather loss	0.132	0.077	1.70	0.193	-0.033	0.402	0.092
Fear scream	-0.048	0.094	-0.51	-0.059	-0.281	0.168	0.607
log Tonic Immobility (s)	0.066	0.118	0.56	0.065	-0.163	0.286	0.575
log Mass (g)	-0.123	0.089	-1.36	-0.156	-0.368	0.072	0.175
λ = 0.000, residual SE=0.063, df=76							
Plasmodium							
log Research effort	-0.081	0.052	-1.54	-0.176	-0.386	0.051	0.125
Alarm call	0.060	0.058	1.04	0.120	-0.108	0.333	0.300
Biting	0.026	0.043	0.59	0.068	-0.159	0.289	0.551
Wriggle	-0.011	0.029	-0.38	-0.044	-0.266	0.183	0.704
Feather loss	0.048	0.051	0.93	0.107	-0.121	0.325	0.352
Fear scream	0.066	0.061	1.07	0.123	-0.105	0.339	0.285
log Tonic Immobility (s)	-0.083	0.079	-1.05	-0.121	-0.337	0.107	0.297
log Mass (g)	-0.038	0.078	-0.48	-0.055	-0.277	0.172	0.626
λ = 0.258, residual SE=0.0447, df=76							

I García-Longoria L, Møller AP, Garamszegi LZ (2014) Host escape behaviour and blood parasite infections in birds. Behav Ecolo 25:890-900.

II García-Longoria L, Møller AP, Balbontín J, de Lope F, Marzal A. Do malaria parasite manipulate the escape behaviour of their avian host? An experimental study. Parasitology. *Under review*.

III Marzal A, García-Longoria L, Callirgos JMC,Sehgal RNM (2015) Invasive avian malaria as an emerging parasitic disease in native birds of Peru. Biol invasions 17: 39-45.

IV García-Longoria L, Hellgren O, Bensch S (2014) Molecular identification of the chitinase genes in *Plasmodium relictum*.Malar J 13:239.

V García-Longoria L, Hellgren O, Bensch S, de Lope F, Marzal A. Detecting transmission areas of malaria parasites in a migratory bird species. Parasitology. *Under review*.

VI García-Longoria L, Coon CAC, Martin LB, Magallanes S, de Lope F, Marzal A. Malaria infection affect negatively feather growth rate in house sparrows. Manuscript.