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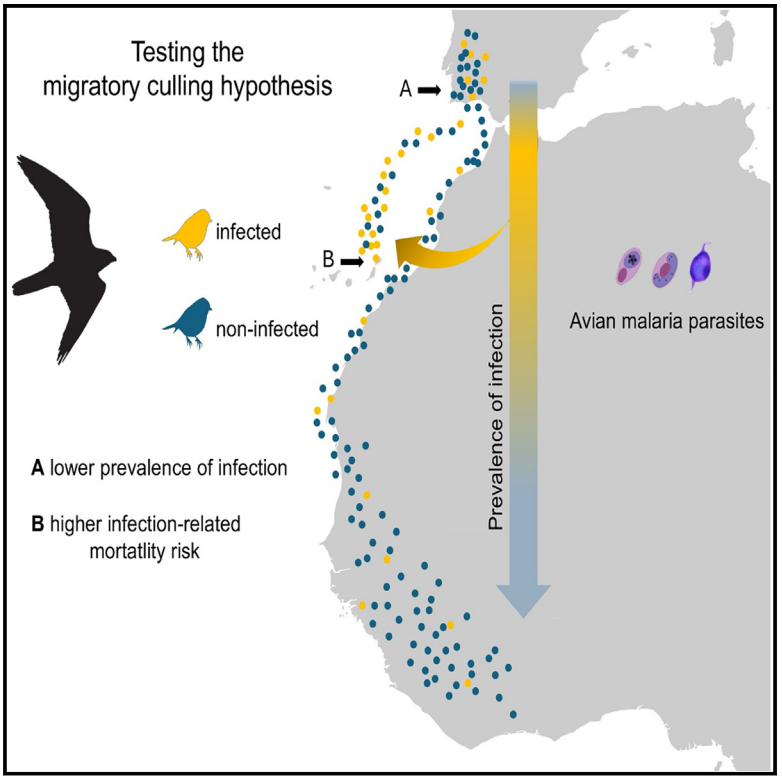
Graphical abstract



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In brief

Wildlife microbiology; Ecology; Ornithology; Evolutionary biology

Highlights

- Infected birds are predicted to die during long-distance migration
- Avian malaria prevalence was higher midway than before crossing to Africa
- This higher prevalence occurred in birds that drifted off course and were hunted by predators
- We provide empirical support to the migratory culling hypothesis

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Does malaria infection increase the risk of predation-related mortality during bird migration?

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SUMMARY

The migratory culling hypothesis posits that infected individuals are less likely to survive long-distance migration due to physiological and behavioral effects, but this lacks empirical evidence. Here, we tested this hypothesis by sampling 357 passerines from 11 species during their autumn migration to wintering grounds in two different areas, i) at a stopover in southern Spain, and ii) in the Canary Islands, where they were drifted and preyed upon by Eleonora's falcons while *en route* to the southern Sahara. Molecular detection of infections by *Plasmodium*, *Haemoproteus,* and *Leucocytozoon* was conducted on bird samples. A higher prevalence of both *Plasmodium* and *Haemoproteus* was observed in birds preyed upon by falcons. While a complete understanding of the mechanistic effects of haemosporidian infections on migration performance needs experimental validation, our approach suggests that infection reduces migration success by increasing mortality due to route deviations and/or predation.

INTRODUCTION

Avian malaria and malaria-like parasites of the genera *Plasmodium*, *Haemoproteus*, and *Leucocytozoon* are protozoa capable of infecting species of every avian order worldwide, causing severe effects on their body condition, reproductive success, and survival.[1–5] Parasite infection causes numerous alterations, mainly related to the lysis of red blood cells, which generates hemorrhages and blockage of capillaries in different inner organs and decreases the oxygen-binding capacity of hemoglobin, thus increasing red blood cell production rate.[5,6] These symptoms are most severe during the acute phase of the infection.[2,7] However, according to other studies, avian malaria infections seem to be asymptomatic in birds,[8] especially during the chronic phase of infection, which is characterized by low parasitemia that can persist throughout the host's lifetime.[7] However, the chronic phase of infection can also have important deleterious effects,[6,9,10] but detecting these effects often requires the experimental manipulation of parasite loads.[11] For example, chronic infections affect multiple host traits, such as behavior,[12,13] feather growth,[14] reproductive success,[15,16] body condition,[17] and survival.[11,18] However, the impact of parasite infection on bird migration is not so straightforward. For example, chronic infections by haemosporidians have been found in many successful long-distance migratory birds,[2,19] and negative associations between parasite prevalence and migratory behavior are not always reported[20–22] or they have only been found in specific fractions of the population (e.g., juvenile individuals[6,23]). This questions the extent to which the effects of infection by haemosporidians compromise migratory performance and success.[24]

Nonetheless, it is expected that infection by haemosporidians imposes an important selective pressure on migratory birds. Among other factors, the impact of infection on hosts' physiology might depend on parasite identity[25] and the migration strategies of birds, including, for instance, short- or long-distance migration, spring or autumn migration, as well as the course of migration, including the timing of departure, and the number and duration of stopovers.[26,27] For example, Garvin et al.[26] observed lower body mass and fat scores in some Neotropical migratory passerines infected by *Haemoproteus* during spring migration, a pattern that was not found by Ágh et al.[27] in European robins (*Erithacus rubecula*) during autumn migration. However, the later study found that infected juveniles arrived later than non-infected ones, especially those infected by *Haemoproteus*. Likewise, yellow wagtails (*Motacilla flava*) had worse body conditions when infected with *H. motacillae* during spring and autumn migration compared to infection with *H. anthi*.[25] Furthermore, infected young male Eurasian blackcaps (*Sylvia atricapilla*) have difficulties in maintaining the redox balance during stopovers, which is related to a greater preference for foods rich

in antioxidants over those rich in fat, which could compromise the acquisition of the amount of fat necessary to complete a successful long-distance autumn migration.[28]

Changes in migratory behavior associated with these parasitic infections have also been reported, including shorter migration distances,[22] delays in the onset and arrival at spring and autumn destinations[27,29,30] and changes in the ecology of the stopovers, such as increases in the length of stays and delays in the departure of infected individuals.[31] Haemosporidian infections may also alter other important behaviors such as orientation during migration[32] and responses to threatening situations. For instance, it has been suggested that symptoms associated with chronic haemosporidian infections may increase the risk of predation[33] (but see García-Longoria et al.[34]). An experimental study revealed that infection by these parasites interferes with exploration and predation risk behaviors in the blackcap (*Sylvia atricapilla*) before its first migration. Infected blackcaps initiated exploration earlier and showed reduced anti-predator behavior, compared to uninfected ones.[13]

This evidence suggests that infection by haemosporidians adds extra costs to an already physiologically demanding and risky task such as migration. The very few studies that have been able to compare the prevalence of avian haemosporidian infections through the full annual cycle of migratory birds found a lower prevalence during the mid-migration, both during spring and autumn passage and late in winter, compared to the breeding season and the onset of autumn migration.[35] Likewise, the prevalence of haemosporidians in a passerine bird was higher during migration just before they left Europe than in their African wintering grounds.[36] In another bird species from America, overall haemosporidian prevalence increased upon arrival at the breeding grounds in spring, peaked during the breeding season and decreased as fall migration progressed.[37] In addition, Slowinski et al.[38] found a higher prevalence of haemosporidians in resident populations compared to migratory counterparts when they simultaneously sampled birds in a shared area at the beginning of winter. One possible explanation for this pattern is migratory culling, which refers to the selective mortality of severely infected individuals along the flyway.[39,40] Although there is theoretical support for the idea that haemosporidian infections can negatively impact migration performance through different physiological mechanisms,[28,32] and theoretical models suggest that migratory culling is a critical factor in shaping migration-parasite infection outcomes,[24,41–43] there is still very little empirical evidence to support these ideas in haemosporidian-infected birds. Migratory culling has been documented in Monarch butterflies (*Danaus plexippus*) that were experimentally infected with a protozoan.[39] In addition, during migration across North America, parasite prevalence declined as monarchs progressed southward.[40] Migratory culling has also been claimed when finding lower migratory performance of GPS-collared Bewick's swans (*Cygnus columbianus*) naturally infected with avian influenza.[44] Infected Bewick's swans showed delayed migration, departing from their wintering grounds more than a month later than their uninfected counterparts. This was associated with infected swans traveling shorter distances and refueling at reduced rates.[44] Migratory culling was indirectly tested in dark-eyed juncos (*Junco hyemalis*) by comparing haemosporidian parasite infections between resident and migratory populations.[38] At the beginning of the winter, dark-eyed juncos from the sedentary population showed a higher prevalence of haemosporidians than those from the migratory population, while no differences in parasitaemia were found among infected individuals.[38] However, to the best of our knowledge, the migratory culling hypothesis, particularly the prediction of increased infection-related mortality risk during migration, has not been empirically tested in free-living migratory birds due to the unfeasibility of sampling individuals that have not survived to complete their migration.

We used a unique study system focusing on one of the most critical stages of the autumn migration of long-distance trans-Saharan migratory birds: circumventing the Sahara Desert by flying over the ocean along the African coast. In a previous study, we unraveled the mechanism by which some of these birds (up to 1 million per day, mostly passerines) departing from the south of the Iberian Peninsula reach the Canary Islands, diverted by the Atlantic trade winds from their intended path along the western African coastline to their wintering grounds[45] (Figure 1). Drifted birds face continuous flight over the open sea for extended periods (mean = 20h ± 4.16 SD) with no chance of landing before reaching the archipelago.[45] The eastern Canary Islands hold colonies of Eleonora's falcon (*Falco eleonorae*), a raptor specialized in hunting these migratory birds over the ocean. Under conditions of intense migratory flux and strong easterly winds, Eleonora's falcons accumulated prey in larders around the nest sites, which allowed us to sample birds that had not successfully completed their migration.[46,47] We hypothesized that infected passerine birds will likely be more prone to be blown off course and predated during their long-distance migration due to the physiological and/or behavioral costs imposed by infection. To test this hypothesis, we compared the prevalence of infection by the blood parasite genera *Plasmodium*, *Haemoproteus,* and *Leucocytozoon* between birds sampled during active autumn migration 1) at a stop-over in the South of the Iberian Peninsula before starting the crossing to African wintering grounds and 2) when killed by falcons in their accidental passage through the Canary Islands. If infected birds are more likely to be diverted from their route and hunted, we expect the prevalence to be higher on the islands than on the Iberian Peninsula.

RESULTS

A high diversity of parasite lineages is on the move

Out of the 357 birds sampled, 152 were infected by at least one parasite genus (prevalence = 0.43 ± 0.03 SE) (Table 1). On the Iberian Peninsula, the overall parasite prevalence (three parasite genera pooled) was 0.34 ± 0.03 SE (n = 62 infections; ranging from 0 to 42.31% for the different avian species depending on the parasite genus), while in the Canary Islands, it reached 0.52 ± 0.03 SE (n = 90 infections; ranging from 0 to 60%) (Table 1). *Plasmodium* spp. was found infecting 69 birds (29 and 40 in the Iberian Peninsula and the Canary Islands, respectively), *Haemoproteus* spp. was found in 66 birds (28 and 38, respectively) and *Leucocytozoon* spp. was found infecting 17 birds (5 and 12, respectively) (see Table 1). In addition, three individuals sampled on the Canary Islands presented mixed-genus infections by

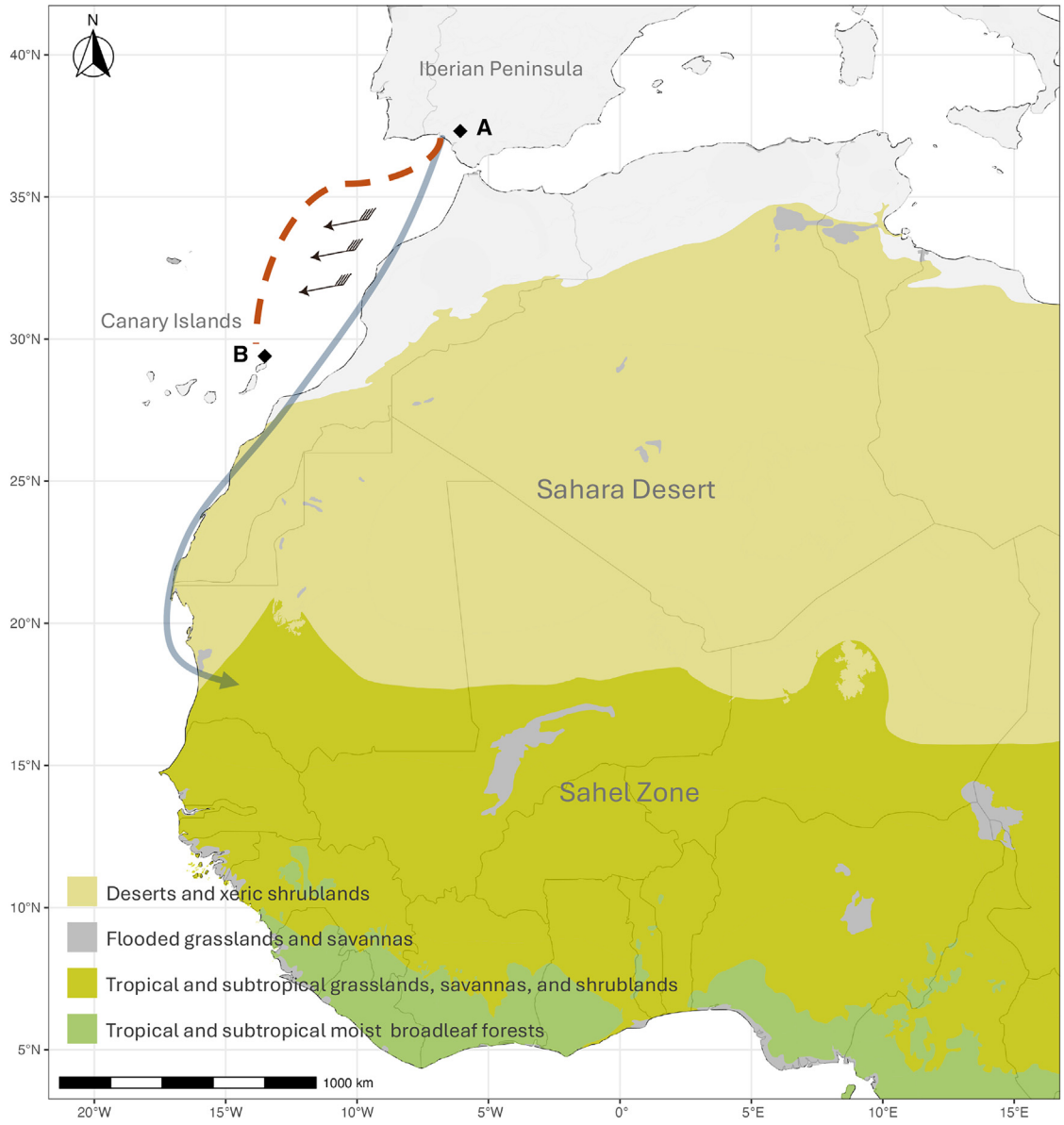

Figure 1. Migratory route of trans-Saharan birds departing from the south of the Iberian Peninsula

This route along the West African coast toward their wintering grounds in the Sahel region is indicated by a blue arrow, while the orange dashed line represents the path taken by birds diverted by the northeast trade winds (three black arrows).[45] The locations where we sampled the birds (A: Palomares del Río, southern Iberian Peninsula, and B: Alegranza islet, Canary Islands) are marked with black squares. The color legend shows the four different biomes highlighted, based on the Nature Conservancy 2019 classification.[48]

Plasmodium/*Haemoproteus* parasites. The common grasshopper warbler (*Locustella naevia*) was the only species not infected by any parasite at any location, although only nine individuals were analyzed.

A total of 45 different parasite lineages were identified in the 11 bird species sampled at both locations (17 *Haemoproteus* spp., 17 *Plasmodium* spp., and 11 *Leucocytozoon* spp.) including two lineages belonging to *Plasmodium* sp. (SYCAN02, GenBank: PQ129498) and *Leucocytozoon* sp. (FICHYP01, GenBank: PQ129497) described here (Figure 2). The most common parasite lineage isolated from birds was *Plasmodium relictum* SGS1 (21.71% of total infections, $n = 152$), followed by *Haemoproteus* PFC1 (15.13%). Phylogenetic relationships showed that the *Plasmodium* lineage SYCAN02 clusters with high confidence within the *Plasmodium relictum* group. The *Leucocytozoon* lineage FICHYP01 clusters with four lineages that do not have a species assigned. Three of these four lineages have only been found in Sub-Saharan Africa, while the fourth lineage has been found in Europe, according to MalAvi database.[49] Lineage diversity was similar between locations, with 9 and 12 lineages of *Plasmodium*,

Table 1. Prevalence of infection (%) in birds included in this study

	N sampled		Prevalence					
			Plasmodium		*Haemoproteus*		*Leucocytozoon*	
Bird species	IB	CI	IB	CI	IB	CI	IB	CI
Acrocephalus scirpaceus	26	3	7.69 (2)	0	42.31 (11)	0	3.85 (1)	0
Ficedula hypoleuca	32	67	0	8.95 (6)	18.75 (6)	35.82 (24)	3.13 (1)	4.48 (3)
Hippolais polyglotta	8	12	37.5 (3)	8.33 (1)	12.5 (1)	58.33 (7)	0	0
Lanius senator	3	2	0	50 (1)	33.33 (1)	50 (1)	0	0
Locustella naevia	4	5	0	0	0	0	0	0
Luscinia megarhynchos	11	8	27.27 (3)	37.5 (3)	18.18 (2)	12.5 (1)	0	0
Muscicapa striata	11	10	36.36 (4)	60 (6)	9.09 (1)	10 (1)	0	10 (1)
Phoenicurus phoenicurus	20	11	10 (2)	0	0	9.09 (1)	15 (3)	18.18 (2)
Phylloscopus trochilus	0	3	–	0	–	0	–	33.33 (1)
Sylvia borin	21	2	14.29 (3)	0	23.81 (5)	0	0	0
Sylvia cantillans	2	8	0	37.5 (3)	0	0	0	12.5 (1)
Sylvia communis	46	42	26.09 (12)	47.62 (20)	2.16 (1)	7.14 (3)	0	9.52 (4)
Total	184	173	15.76 (29)	23.12 (40)	15.22 (28)	21.96 (38)	2.72 (5)	6.94 (12)

Data include values for *Plasmodium*, *Haemoproteus,* and *Leucocytozoon* parasites in the bird species sampled at both the Iberian Peninsula (IB) and the Canary Islands (CI). The number of infected individuals of each bird species is shown in brackets.

11 and 9 of *Haemoproteus*, and 5 and 8 of *Leucocytozoon* in the Iberian Peninsula and the Canary Islands, respectively (Figure 2; Table S1). Nine parasite lineages were shared between the two locations (four *Plasmodium*, three *Haemoproteus*, and two *Leucocytozoon* lineages, Figure 2), and seven of these were found in the same bird species: *Haemoproteus* LULU1 infecting nightingales (*Luscinia megarhynchos*), HIPOL1 in melodious warblers (*Hippolais polyglotta*), PFC1 in European pied flycatchers, *Plasmodium* GRW11 and SYAT05 in common whitethroats and SGS1 in spotted flycatchers (*Muscicapa striata*), and *Leucocytozoon* BT1 in common redstarts (*Phoenicurus phoenicurus*). Other lineages were exclusively found in single individuals of some bird species, particularly those with lower sample sizes, or captured in a single location (see Table S1).

Parasite prevalence was higher in birds blown off course and hunted

When considering the 11 bird species sampled in both locations, the prevalence of *Plasmodium* was significantly higher in the Canary Islands than in the Iberian Peninsula (LR test, *Plasmodium*: $\chi^2 = 4.86$, df = 1, $p = 0.02$). The prevalence of both *Haemoproteus* and *Leucocytozoon* was also higher in the Canary Islands than in the Iberian Peninsula, although this difference was only marginally significant (LR test, *Haemoproteus*: $\chi^2 = 3.15$, df = 1, $p = 0.07$; *Leucocytozoon:* $\chi^2 = 3.21$, df = 1, $p = 0.07$). Furthermore, when considering only the two bird species with the largest sample sizes, results were consistent, and in the Canary Islands the prevalence of *Plasmodium* and *Haemoproteus* was significantly higher (LR test, *Plasmodium*: $\chi^2 = 6.89$, df = 1, $p = 0.008$; *Haemoproteus*: $\chi^2 = 4.65$, df = 1, $p = 0.03$) (Figure 3). The prevalence of *Leucocytozoon*, was again marginally significant (LR test, $\chi^2 = 3.40$, df = 1, $p = 0.06$) but this model did not perform well because the random effects covariance matrices of the fitted model were singular, i.e., the variances of one or more linear combinations of effects were (close to) zero. This is likely due to the low prevalence of *Leucocytozoon* infection found in most species (Table 1).

Birds' age, sex, and sampling date did not affect the probability of infection by *Plasmodium* (age $\chi^2 = 1.21$, df = 1, $p = 0.27$; sex $\chi^2 = 0.01$, df = 1, $p = 0.93$; date $\chi^2 = 0.79$, df = 1, $p = 0.37$) and was only marginally significant for the sampling date in the case of *Leucocytozoon* (age $\chi^2 = 0.10$, df = 1, $p = 0.75$; sex $\chi^2 = 0.07$, df = 1, $p = 0.79$; date $\chi^2 = 3.34$, df = 1, $p = 0.07$). In the case of *Haemoproteus*, the birds' sex and sampling date had no effect on the probability of infection (sex $\chi^2 = 2.18$, df = 1, $p = 0.14$; date $\chi^2 = 0.51$, df = 1, $p = 0.48$). In contrast, their age did ($\chi^2 = 14.65$, df = 1, $p < 0.001$) and older birds (born before 2019, that is, at least in their 2 nd year) had a higher prevalence than yearlings.

DISCUSSION

We molecularly screened for blood parasites in European passerines during their autumn migration to wintering grounds. Sampling was conducted at a stopover in southern Spain and among birds drifted and preyed upon by Eleonora's falcons in the Canary Islands *en route* to the southern Sahara. Our findings reveal a considerable diversity of blood parasite lineages in these birds, accompanied by significant differences in infection prevalence across the sampling locations. The prevalence of haemosporidian parasites in birds was lower at the start of the crossing to Africa than in those that were drifted by the wind and hunted by avian predators in an accidental endpoint. Our findings suggest that infected birds may be more likely to stray from their intended migratory path and become preyed upon, providing support for the migratory culling hypothesis.[39] Although we do not know the specific mechanisms underlying the infection effects on the bird's physiology and behavior, our study highlights the potential impact of infection on bird migration. The hunting

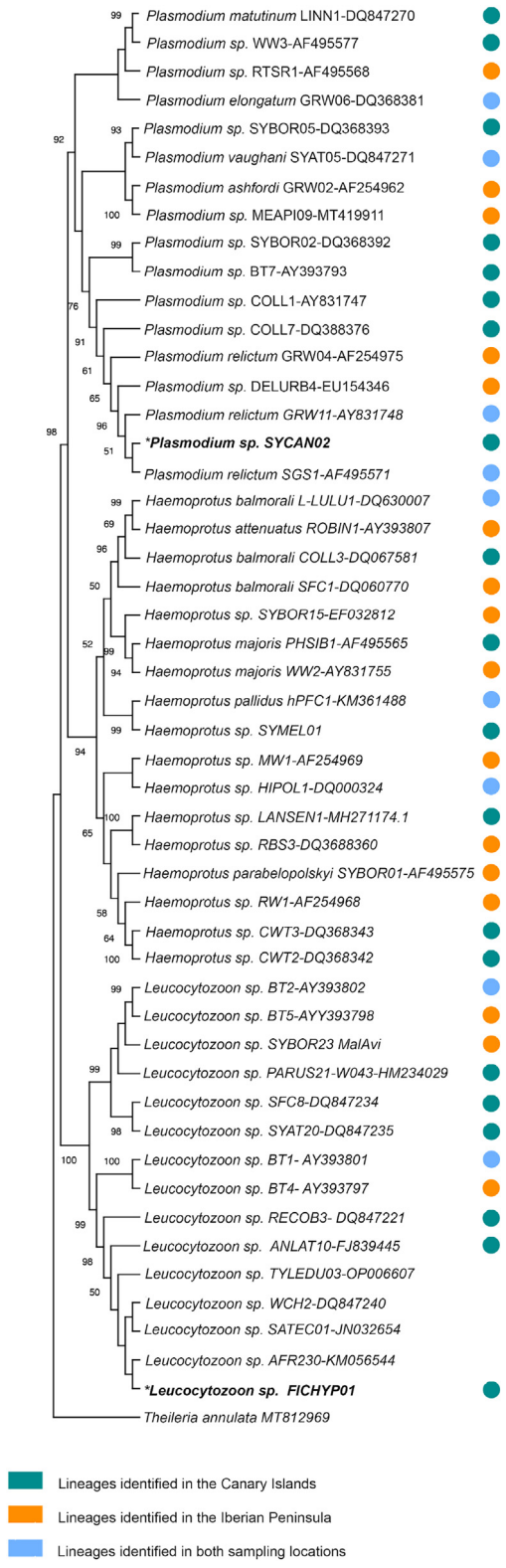

Figure 2. Phylogenetic tree of parasite lineages found in birds

The high diversity of lineages from the genera *Plasmodium*, *Haemoproteus*, and *Leucocytozoon* is interspersed among migratory birds sampled in the Iberian Peninsula (orange dots) and the Canary Islands (green dots). Nine lineages were found in both locations (blue dots). The bootstrap consensus tree was inferred from 1.000 replications for the parasite lineages found in this study, as well as the four closest sequences from known morphospecies to the *Leucocytozoon* lineage deposited in MalAvi.[49] The two lineages described here are bolded and highlighted with an asterisk (*). Only boostrap values above 50 are shown. *Theileria annulata* was included as an outgroup.

behavior of falcons allowed us access to the otherwise unfeasible sampling of wild birds that did not manage to complete their long-distance migration and thus, test the hypothesis of a blood parasite infection-mediated filter during migration. Our observational study would therefore provide empirical evidence of increased mortality risk in infected migratory individuals using different bird species infected by haemosporidians.

Previous studies have found a general pattern of lower prevalence of haemosporidian infections in birds during mid-migration and late winter compared to the breeding season. For example, in the garden warbler (*Sylvia borin*), a sub-Saharan long-distance migrant, the overall haemosporidian parasite prevalence was higher on breeding grounds and at the onset of autumn migration, than during migration, both during spring and autumn passages.[35] This pattern was explained by host-parasite interactions and specific parasite transmission strategies, although parasite-induced increased mortality during migration was also considered.[35] *Plasmodium* prevalence was also higher in the aquatic warbler (*Acrocephalus paludicola*), a sub-Saharan migrant, soon before leaving Europe during fall migration than on the wintering grounds.[36] These authors suggest that infected warblers either overcame the infection or perished during autumn migration.

Haemosporidian infections have demonstrated the capacity to influence behavioral traits among avian hosts, including migratory behavior[22,27,30,31] and responses to threatening situations such as predation risk.[33] While the impact of malaria infection on risk-taking behaviors is still an area of limited evidence, there are conflicting outcomes across studies and avian species. For instance, an increase in risk-taking behaviors among infected individuals was found in house sparrows (*Passer domesticus*)[34] and nightingales.[50] However, infected yellow wagtails exhibited decreased risk-taking behaviors[51] and infected blackcaps showed weaker sentinel behavior and reduced alarm calls than uninfected counterparts,[13] while no discernible differences were found between infected and uninfected collared flycatchers (*Ficedula albicollis*).[52] The physiological costs associated with the infection *per se* and the immune system activation[53] would make the most affected or immunocompromised individuals more susceptible to predation.[33] When this overlaps with another extremely energy-demanding activity such as migration, the resulting trade-offs involving hemoglobin concentration, fat stores, oxidative balance, and immune defenses, among others,[28,54,55] may further increase individuals' mortality risk. Evidence for higher predation risk of infected individuals was provided by Furey et al.[56] in a different host-parasite system, who found that sockeye salmons (*Oncorhynchus nerka*) infected with IHN virus had a greater chance of predation during migration than uninfected salmon. Our findings propose a potential link between haemosporidian parasite infections and

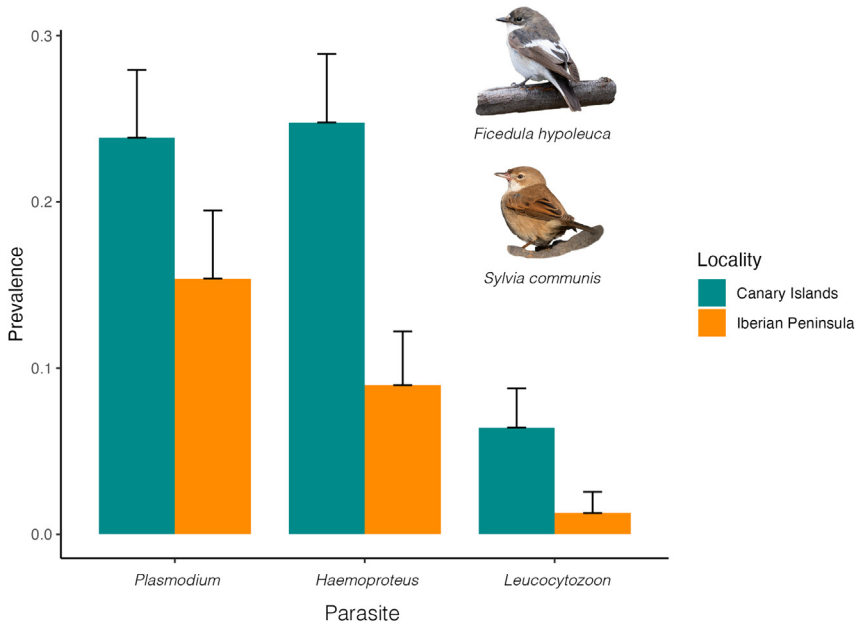

Figure 3. Prevalence of infection in birds from the two study areas

Data from both the European pied flycatcher (*Ficedula hypoleuca, n* = 32 and 67 individuals from the Iberian Peninsula and the Canary Islands, respectively) and white throat (*Sylvia communis, n* = 46 and 42 from the Iberian Peninsula and the Canary Islands, respectively) is pooled. Prevalence of infection in birds blown off course and preyed upon by falcons at the Canary Islands are shown in green while those birds sampled in the Iberian Peninsula before crossing to Africa are represented in orange. Data are represented as mean prevalence +/− standard error. Photos: Aitor Gil Guruceaga.

heightened mortality of birds during migration, specifically through increased predation risk.

We found a generalized pattern of higher prevalence of infection by *Plasmodium* and *Haemoproteus,* and marginally by *Leucocytozoon* in those birds that did not complete their migration, irrespective of the birds' age and sampling date. Only the prevalence of *Haemoproteus* was related to birds' age, with older birds showing higher prevalence. It could be argued that the higher prevalence of infection we found on the Canary Islands is associated with the dynamics of parasites coupled with host's physiology, such as recrudescence or relapses of parasite infections during challenging annual-cycle periods such as migration.[5,57] However, our sampling period was short, and all birds sampled at both locations were in active migration with similar energy demands, so notable differences in prevalence are not expected after about a day of sustained flight from the Iberian Peninsula to the Canary Islands.[45] Alternatively, our results could be explained by the capture of individuals from different populations, not connected through the same migratory route. However, the occurrence at both the stop-over site and the accidental endpoint of birds originating from multiple European breeding populations (see STAR Methods) buffers potential geographical variation and local effects affecting the diversity and prevalence of parasites.

Data on the prevalence of haemosporidians in the bird species analyzed in this study, or in other trans-Saharan migratory passerines sampled in the Iberian Peninsula at the onset of autumn migration, is limited. Previous studies reported parasite prevalence in the common reed warbler (*Acrocephalus scirpaceus*) ranging from 0.25[58] to 0.81.[59] Additionally, prevalence rates of 0.25 were found in the aquatic warbler[36] and 0.06 in the willow warbler (*Phylloscopus trochilus*).[58] The mean overall prevalence across these studies, based on birds sampled over different years, was 0.32 ± 0.02 (see Table S2). Discrepancies in prevalence data were even more pronounced depending on the parasite genus (Table S2), suggesting that baseline data on haemosporidian prevalence is highly heterogeneous and cannot be reliably extrapolated across species or years. It is important to note that when sampling individuals at breeding grounds, parasite prevalence may be heavily influenced by local conditions.[60] However, by sampling birds at migratory stopovers, where animals from various origins stay for short periods, we gain a broader picture of parasite prevalence on a larger scale. Therefore, although our approach is relatively simple, it provides a useful comparison of prevalence data for the same species between two locations over a short time period. Nevertheless, it would be advisable to gather a larger sample of individuals from different areas of the Iberian Peninsula to gain a more accurate understanding of infection prevalence at the beginning of autumn migration along this route.

All 45 haemosporidian lineages we identified have been recovered in Europe in various bird species, and 32 of them were also reported in Africa[49] (see Table S1). Two lineages were particularly prevalent. The generalist *Plasmodium* lineage SGS1 has been reported in migratory birds from Africa, Europe, Asia, and North America, including 43 species from 9 families and 3 orders (Charadriiformes, Gruiformes, and Passeriformes).[49,61] On its part, the *Haemoproteus* lineage PFC1 has been previously described in migratory birds of the genus *Ficedula* (*F. hypoleuca* and *F. albicollis*) in Europe and Asia, but not in Africa.[49] Despite the extensive diversity of parasite lineages described in bird hosts[49] and the likelihood that the bird species sampled here breed in different European countries, the finding that nine parasite lineages were shared between birds sampled in the Iberian Peninsula and the Canary Islands, seven of which were even found in the same bird species (Figure 2; Table S1), further supports the idea that these birds belong to a common migratory pool.

A growing body of ecological theory underscores the potential for migration to serve as a mechanism for decreasing parasite prevalence within populations.[43,54,62–65] A plausible avenue for this phenomenon involves the confluence of physiological demands related to both migration and infection,[28] culminating in the sustained removal of infected individuals from the population

through migratory culling.[39] Previous indirect evidence[35–38] and the results of this study might support the idea that infection by avian haemosporidians reduces migration success and thus, increases infection-related mortality of migrants. It has recently been suggested that *Haemoproteus* infections may cause brain disorders and alter host behavior, favoring deviations from their inherited migration direction, and ending up outside their normal range.[32] These vagrant birds could be considered evolutionary dead ends due to their increased mortality rates but might also contribute to parasite expansion to wider areas and host ranges.[66] Like in the case of the migratory culling hypothesis, there is yet no empirical evidence for such an interesting possibility, as it requires collecting vagrant, dead individuals. Original study systems such as that used here would allow to sampling of such birds in future studies.

Limitations of the study

Our study is observational and, therefore, we cannot unequivocally conclude that it is the infection by haemosporidian parasites that causes birds to deviate from their route and be at greater risk of predation. Except for blood samples, we did not obtain additional samples that would allow us to determine the physiological state of the birds. Future research could be further enhanced in various ways. First, sampling birds in Africa shortly after completing this same migration route could clarify whether uninfected birds or those with lower parasite loads are those who have managed to complete the migration successfully. If infection rates are higher in birds that were drifted compared to those that reached their wintering destination, it would suggest that haemosporidian infection has a direct effect on migration performance or success. Second, sampling birds in the Canary Islands that have been diverted from their migratory route but not hunted by a predator could clarify the role of infection in risk-taking or escape behaviors, as well as the role of a predator in the probability of parasite dispersal. Because the number of birds that arrive alive at the breeding site of falcons (Alegranza islet) in the Canary Islands is very low, and they are typically in very poor condition, sampling should be conducted on other major islands of the archipelago (e.g., Lanzarote and Fuerteventura), where these migratory birds may stop to rest and refuel. If the infection rate is higher in predated birds compared to live ones, it would provide strong evidence for the effect of parasite infection on predation. Third, taking additional biometric measurements and samples (e.g., hemoglobin levels) of sampled individuals, including brain tissue samples from hunted birds, would shed light on the physiological effects of infection in natural settings and how these may contribute to migratory culling and/or parasite spread in migratory birds.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Laura Gangoso (lgangoso@ucm.es).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- Parasite lineages data have been deposited in GenBank and are publicly available as of the date of publication. Accession numbers are listed in the key resources table. Prevalence data have been deposited at Zenodo and are publicly available as of the date of publication. The DOI is listed in the key resources table.
- All original code is available in this article's supplemental information.
- Any additional information required to reanalyze the data reported in this article is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

Conceptualization, L.G., J.M.P, and J.F.; methodology, L.G., J.M.P., and J.F.; formal analysis, L.G., C.S.C. ,and M.R.L.; investigation, L.G.; resources, L.G. and J.F.; data curation, L.G. and M.R.L.; writing – original draft, L.G.; writing – review and editing, L.G., C.S.C., J.M.P, M.R.L., and J.F.; supervision, L.G., J.M.P., and J.F.; funding acquisition, L.G. and J.F.

DECLARATION OF INTERESTS

The authors declare no competing interests.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Bird blood	DNA	N/A
Deposited data		
Raw data	This paper, Zenodo	https://doi.org/10.5281/zenodo.13118546
Leucocytozoon lineage FICHYP01	This paper, GenBank	PQ129497
Plasmodium lineage SYCAN02	This paper, GenBank	PQ129498
Software and algorithms		
R software version 4.4.0	R Core Team	https://www.r-project.org/

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Bird sampling

We used blood samples from wild birds from two sources: (1) birds captured alive in the Iberian Peninsula (n = 184 birds of 11 species) and (2) birds killed by Eleonora's falcons in the Canary Islands (n = 173 recent kills comprising 12 bird species, including the 11 species sampled in the Iberian Peninsula; see Table 1). For further details on blood sampling, see below.

The species sampled at both sites were common reed warbler *Acrocephalus scirpaceus*, European pied flycatcher *Ficedula hypoleuca*, melodious warbler *Hippolais polyglotta*, woodchat shrike *Lanius senator*, common grasshopper warbler *Locustella naevia*, common nightingale *Luscinia megarhynchos*, spotted flycatcher *Muscicapa striata*, common redstart *Phoenicurus phoenicurus*, garden warbler *Sylvia borin*, subalpine warbler *Sylvia cantillans*, and common whitethroat *Sylvia communis*. Additionally, willow warbler *Phylloscopus trochilus* was sampled only in the Canary Islands.

The sex of the birds was determined molecularly (see below), while their age was assigned based on plumage characteristics following EURING age codes (2 categories). Category 3 refers to birds hatched during the current calendar year – yearlings – with partial or complete juvenile body plumage. Category 4 refers to birds hatched before the current calendar year, with the exact birth year unknown. Birds' age and sex did not influence the probability of infection by *Plasmodium* or *Leucocytozoon*. For *Haemoproteus*, sex had no effect on infection probability; however, age did, with older birds (hatched before 2019, and therefore at least in their second year) showing a higher prevalence than yearlings.

All experiments conform to the relevant regulatory standards. Permits for field work in the Iberian Peninsula were granted by the Spanish Regional Authority (Junta de Andalucía: permission number: 02/05/2018/062). Permits for field work on the Canary Islands were granted by Regional (Gobierno de Canarias; permission numbers: 343/2015 and 278/2017) and Local (Cabildo de Lanzarote; permission number: 2019-1799) Authorities.

METHOD DETAILS

Study areas and field sampling

We sampled birds from 18th August to 9th October 2019 simultaneously at a stop-over just before crossing to Africa (Palomares del Río, Sevilla, southwestern Spain) and at an accidental arrival site in the middle of the flyway (Alegranza islet, Canary Islands; 1050 ha, 289 m above sea level). Ringed birds found in the Alegranza islet over the years confirmed they came from European countries including Portugal, France, Belgium, and Spain.[45] Thus, birds that arrive in the Canary Islands are expected to come from western Europe and they would migrate southwest through the Iberian Peninsula to continue their journey through the West African coast until reaching their wintering quarters in tropical Africa[67,68] (Figure 1). This supports that we are sampling the same bird populations at these two sites.

Sampling was conducted during the peak of autumn migration of trans-Saharan birds, which overlaps the peak of food demands of Eleonora's falcons' nestlings. We focused on the bird species that Eleonora's falcons' primarily prey on in the Canary Islands.[47]

Blood sampling

Birds in the Iberian Peninsula were captured with mist nets at dawn and early morning and were individually marked with a numbered metal ring. Up to 10 μL of blood was sampled from the jugular vein with a sterile syringe and preserved in Eppendorf tubes filled with absolute ethanol. On the Canary Islands, killed birds were partially beheaded with a sterile scalpel and a blood sample of ca. 10 μL was obtained with a sterile syringe and immediately preserved in Eppendorf tubes filled with absolute ethanol. These birds were

sampled at Eleonora's falcons' larders coinciding with the visits to the nests done during regular population monitoring. We left the sampled kill in the same place it was found for later consumption by the falcons. All samples were stored at -20°C until molecular analyses.

Molecular analysis

Genomic DNA from blood samples was extracted using a DNeasy blood and tissue kit (Qiagen, Valencia, California, USA) following the manufacturer's protocol for tissue extraction to maximize the DNA yield from clotted samples. We determined the presence and identity of *Plasmodium, Haemoproteus,* and *Leucocytozoon* parasites in birds following Hellgren et al.[69] The presence of amplicons was verified in 1.8% agarose gels, and positive samples were sequenced using the BigDye® technology (Applied Biosystems) at the Macrogen sequencing service (Macrogen Inc., Madrid, Spain). Sequences were edited using the software Geneious v. 2020.0.3[70] and assigned to parasite lineages after comparison with the GenBank (National Centerre for Biotechnology Information) and MalAvi[49] databases using the BLAST tool.[71] To ensure accuracy, we conducted DNA amplification and sequencing of the barcoding fragment of the cytochrome b gene twice from independent reactions for all sequences that differed by at least one base from previously isolated lineages. This was done to eliminate any possibility that the putative lineages described here were a result of degraded DNA or sequencing errors. Sequences identified in this study are GenBank: PQ129497, PQ129498. Sex was determined following Griffiths et al.[72,73]

Phylogenetic analysis

We included all the lineages found in both sampling sites, that is, the Iberian Peninsula and the Canary Islands, as well as the four closest sequences to the *Leucocytozoon* lineage described here. We also included *Theileria annulata* (MT812969) as an outgroup. Multiple alignments were carried out with Clustal W[74] in CIPRES.[75] Tamura-Nei was the best substitution model in the analyses carried out using jmoldeltest 2.1.10[76,77] in CIPRES. The phylogenetic tree was generated using a maximum likelihood approach, a Tamura-Nei model and 1.000 bootstraps in MEGA11: Molecular Evolutionary Genetics Analysis version 11.[78]

QUANTIFICATION AND STATISTICAL ANALYSES

We tested for differences in the prevalence of each parasite genus in birds between the Iberian Peninsula and the Canary Islands using Generalized Linear Mixed Models GLMMs with the package "lme4"[79] that utilized a two-column matrix to represent the number of infected birds of each species in each site in relation to the number of uninfected birds. Analyses were restricted to the 11 bird species sampled in both sites, excluding the willow warbler from the analyses as it was only sampled in the Canary Islands. By using the *cbind* function, *Plasmodium, Haemoproteus,* and *Leucocytozoon* prevalence was individually modeled with binomial error and a logit link function, inherently controlling for sample size. Firstly, we fitted a GLMM for each parasite genus including all birds, where the bird species was included as a random effect and the site as the only fixed factor. Secondly, to reach better statistical fit and reliable estimates of the prevalence of infection,[80] we fitted similar GLMMs where only those bird species with at least 25 individuals sampled at each locality were included. In this analysis, only the European pied flycatcher (*Ficedula hypoleuca*) and the common whitethroat (*Sylvia communis*) (Table 1) were considered. In addition, to assess the effect of birds' sex, age, and date of sampling on the probability of infection, we fitted GLMMs for each parasite genus with the binomial response (1/0) representing individual infection data. In these models, the locality was also included as a fixed factor and bird species as a random term. Fixed effects were tested using likelihood ratio tests with the package "lmtest".[81] Overdispersion was checked using the overdispFun function. Analyses were performed in R software (v. 4.4.0[82]). All figures were made with the package "ggplot2".[83] Figure 1 also used packages "sf",[84] "ggspatial",[85] "rnaturalearth"[86] and "rnaturalearthdata".[87] All original code is presented in the supplemental information. The data have been deposited at Zenodo: https://doi.org/10.5281/zenodo.13118546.

ADDITIONAL RESOURCES

Data S1. R code used in this study.