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Risk of Infection, Local Prevalence and Seasonal Changes in an Avian Malaria Community Associated with Game Bird Releases

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Abstract: Anthropogenic activities, such as the translocation or introduction of animals, may cause a parallel movement of exotic parasites harboured by displaced animals. Although introduction and/or relocation of animals for hunting purposes is an increasingly common management technique, the effects of gamebird release as a major vehicle for the introduction of parasites into new geographic regions have rarely been reported. We examined the prevalence and distribution of avian malaria parasites infecting resident avian hosts (red-legged partridge *Alectoris rufa*) at a local scale, with a particular emphasis on the effects of releasing farm-reared birds for hunting on the spatial and temporal structure of the parasite community. We collected blood samples from adult partridges from two game estates with partridge releases and two sites without releases over two periods (spring and autumn). We tested the probability of infection and differences in the parasite community in relation to the management model (releases vs. non releases) and sampling period, comparing autumn (when farm-reared birds are released) and spring (after hunting season, when mostly wild birds can be found in the population). We found a high prevalence (54%) of *Plasmodium* spp., and substantial differences in the spatial and temporal distribution of parasite lineages among the populations studied. Some parasite lineages occurred at high frequencies in game estates without introduction of farm-reared partridges, while other lineages were more abundant in game estates with releases than in those without releases. Overall, the prevalence of avian malaria was similar between spring and autumn at non-release sites, whereas in sites with releases, it was higher in autumn than in spring—probably due to artificial restocking with infected farm-reared birds at the onset of the hunting season. In short, humans may be an important agent driving the alteration of the spatial structure of local parasite fauna via the introduction of exotic parasites by gamebird release, which could cause avian malaria outbreaks with severe repercussions for native avifauna.

Keywords: avian malaria; *Alectoris rufa*; host-parasite co-evolution; hunting; farm-reared birds; *Plasmodium*; introduced parasites

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1. Introduction

The anthropogenic actions affecting the distribution and dispersal of animals have a global and continuing influence on the evolutionary course of wild populations. There is now a large body of literature showing remarkable large-scale responses in some animal populations affected by human disturbance [1–3]. Among these perturbations, common practices used today such as the relocation and introduction of animals have played a significant role on the emergence and spread of several diseases [4–10], with significant consequences for wildlife, domestic animals, and humans [11,12]. The human-based spread of infectious agents over new areas jeopardizes wild animal populations by exposure

to exotic pathogens [13–16], some of which may be parasites harboured by introduced animals [17–21]. Avian malaria is a paradigmatic case of a widespread vector-transmitted disease with negative effects on the survival and fitness of many bird species [22–25]. The introduction of parasites into new environments can largely influence parasite evolution, host population dynamics, and host—parasite interactions [26–28], and growing empirical evidence indicates that these kinds of human-induced changes in ecosystems are more common than previously thought [27].

The management of wild species for hunting is probably one of the human activities responsible for much of the alteration occurring at a large scale on wild populations of many species. Introduction and/or relocation of animals is an increasingly common management technique that represents a good example in which humans modify on a large scale the genetic structures, community compositions, and life histories of both parasites and hosts [28–35]. Gamebird releases, particularly those of red-legged partridges (*Alectoris rufa*), provide a suitable model for evaluating the consequences of this kind of wildlife management. The red-legged partridge is a medium-sized *Phasianidae* native to the Iberian Peninsula, France, and Italy, that has suffered a sharp decline in wild numbers during the last decades [36,37]. The reinforcement of wild populations with farm-reared birds is today the most widely used tool in the management of this gamebird, involving millions of released birds over huge areas [32,34,38]. The percentage of released birds that survive after their first "hunting season" (from October to January) is potentially low [39–42], but settlement and successful breeding into the wild of some released individuals has been proven [39]. On the other hand, the habitual conditions of partridge farms (very high densities of animals and increased stress factors) may be particularly favourable for the acquisition of malaria parasites from local avifauna, which has been established afterwards in distant areas where partridges are released in large numbers. Furthermore, releases suddenly increase host density at local scales and, consequently, the number of both intra- and inter-specific contacts between infected and uninfected individuals. Such a phenomenon may indeed be of great conservation concern since it operates on a country-wide scale in the case of Iberian partridges, but also globally in a wide range of species, including fish, mammals, and birds [11]. The importance of hunting management as a pathway for the introduction of new parasites and its potential consequences for wildlife has received increased attention recently [9,12,43], but little has been done to address the importance of farm-reared bird releases as a putative reservoir of infections and avian malaria outbreaks.

The aim of this study was therefore to determine the importance of hunting releases as a way of propagating exotic (i.e., parasites previously non-existent in the receiving host population) avian malaria parasites. Furthermore, we explore potential changes in the prevalence of native parasites as a consequence of releases. We address this question by comparing the parasite community among sites with releases and sites without releases, and between autumn (when farm-reared birds are released) and spring (when mostly wild birds can be found in the population) in both types of sites. Basically, if malaria prevalence (i.e., the percentage of infected birds) and the community composition (the occurrence of each parasite strain) differs between both periods in populations reinforced by releases but not in sites without releases, then the difference may be associated with releases. To further assess the existence of artificial restocking in our populations, we explored the occurrence of partridges with allochthonous mtDNA haplotypes (mainly from *Alectoris chukar*), which is expected to increase after releases due to the liberation of hybrids (*A. chukar* × *A. rufa*) among the captive stock [32,34,42,44,45].

We discuss our findings in relation to how releases may alter the spatial structure of local pathogenic fauna via the introduction of exotic avian malaria parasites, and the potential consequences for the host—parasite system, for wild populations of game species, and for sympatric populations of non-game species.

2. Materials and Methods

2.1. Study Populations and Field Procedure

The study area comprised 7.779 ha located in the Campo de Calatrava region (Central Spain, 38°80′ N, 3°80′ W, 610 m a.s.l.). The habitat was characterized by undulated farmlands aimed at cereal cultivation (mostly barley *Hordeum* spp.), with interspersed patches of olive groves (*Olea* sp.), vineyards, dry annual legume crops (mainly vetch *Vicia sativa*) and sugar beet (*Beta rubra*).

We collected 189 blood samples from adult red-legged partridges between 2003 and 2005 from four game estates (hereafter "sites" A, B, C, and D) 1.8–11.1 km apart from each other (Figure 1 and Table 1). We considered the four sites as independent in our analyses in relation to hosts, because radio-tracked partridges usually have low dispersion rates after releases (average lower than 600 m, see [41]). However, the capacity of some vectors to travel was within this range of distances [46], and parasite transmission could also be vectored by other avian species. Hence, the distance between pairs of sites does not guarantee independence regarding vectors and/or other avian host species movements. The four sampling sites had similar landscape features, climate and the same type of "dry cultivation" without irrigated lands, but differed markedly in game management systems [47]. One of the game estates sampled (site B, 1484 ha.) followed an intensive management model with about 2000 farm-reared partridges released per year, representing 75–90% of the total partridge captures each year. This site maintains the same hunting pressure every year, having an overall hunting bag of 1.35 birds/ha. Site C (548 ha.) showed occasional releasing activity, with hunting pressure adjusted to autumn numbers and an average hunting bag of 0.15 birds/ha. Finally, sites A (3145 ha.) and D (1009 ha.) have followed a traditional management model without captive-bred gamebird restocking for over 10 years. In both sites, hunting pressure was adjusted to autumn numbers, with an average hunting bag of 0.26 and 0.55 birds/ha, respectively [47].

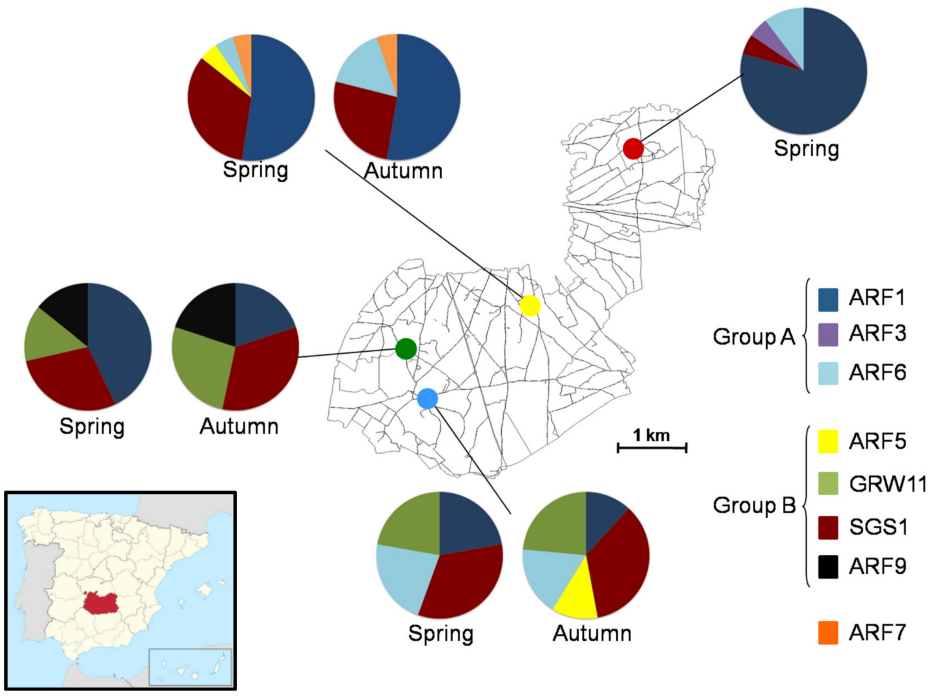

Figure 1. Spatial and temporal distribution of malaria-parasite lineages recorded in four populations of red-legged partridges in Ciudad Real, central Spain (inset shows the location of the Ciudad Real province within the Iberian peninsula). The two main groups of lineages according to Figure 2 are indicated. Pie charts indicate the relative importance of all sequenced *Plasmodium* infections in a given population. The four host sites are labelled by different colours: yellow—game estate A, blue—game estate B, green—game estate C, and red—game estate D. Percentage values are given in Table 1.

Table 1. Number of red-legged partridges infected by each malaria lineage (*Plasmodium* spp.) in four study sites in central Spain.

			Game Estate									
			Site A		Site B		Site C		Site D		Total	
Parasite Taxon	**Lineage**	**GenBank**	**AT**	**SP**	**AT**	**SP**	**AT**	**SP**	**AT**	**SP**	**AT**	**SP**
Plasmodium sp.	ARF1	EU395835	10	11	2	2	3	3	-	11	29.4	51.9
P. relictum	SGS1	AF495571	5	7	6	3	5	2	-	1	31.4	25.0
Plasmodium sp.	ARF3	EU395836	0	0	0	0	0	0	-	1	0.0	1.9
Plasmodium sp.	ARF5	EU395838	0	1	2	0	0	0	-	0	3.9	1.9
Plasmodium sp.	ARF6	EU395839	3	1	3	2	0	0	-	2	11.8	9.6
Plasmodium sp.	ARF7	EU395840	1	1	0	0	0	0	-	0	2.0	1.9
P. relictum	GRW11	AY831748	0	0	4	2	4	1	-	0	15.7	5.8
Plasmodium sp.	ARF9	EU395841	0	0	0	0	3	1	-	0	5.9	1.9
N° of samples			31	39	25	35	21	9	-	29	77	112
N° of infections			19	21	17	9	15	7	-	15	51	52
Prevalence			61.2	53.8	68	25.7	71.4	77.7	-	51.7	66.2	46.4

Note: Autumn and spring sampling periods are designated by AT and SP, respectively. The last two columns show the percentage of infected hosts by each parasite lineage in each of the two periods.

Our analyses were based on two different sampling periods: spring samples (n = 112) were collected in the four populations from wild partridges caught using cage traps with live adult partridges as a decoy [47]; autumn samples (when hunters restocked partridge populations) were taken from 77 hunter-harvested partridges sampled in sites A, B, and C (samples from site D were not available, see Table 1). We were limited in our autumn data collection to hunter-harvested birds rather than birds previous to releases because of the noticeable reticence of hunting managers to allow sampling from farmed stocks. All partridges captured were ringed in order to avoid resampling.

2.2. Avian Malaria Diagnosis

Blood samples were obtained by ulnar venipuncture in live partridges (spring samples) or taken from the heart in hunter-harvested partridges (autumn samples) and stored in 99% ethanol until molecular analysis. DNA was extracted using a standard ammonium acetate precipitation method, and diluted to a working concentration of 25 ng/µL. Samples were screened for the presence of *Plasmodium* and *Haemoproteus* using a widespread nested polymerase chain reaction (PCR) protocol [48] designed to amplify a 479 bp fragment of the mitochondrial cytochrome b gene of both parasite geni. The PCR tests were performed in two separate runs with positive (i.e., DNA from individuals with known malarial infections) and negative (ddH_2O) controls. Pre- and post-PCR work was performed with different materials and in different laboratory sections to avoid contamination. We repeated the protocol three times to confirm negative infections. All samples with positive PCR reactions were successfully sequenced from both ends. We used MicroSpin s-400 HR columns (Amersham Biosciences) to purify PCR products, which were sequenced using the same PCR primers and the Big Dye Terminator Kit (Applied Biosystems, Thermo Fisher Brand, Foster City, CA, USA). The sequencing reactions were purified on standard Sephadex columns and DNA sequences were obtained using an ABI 3130 automated sequencer (Applied Biosystems). Reading data were processed with the ABI PRISM1 Sequencing Analysis Software v3.7 (Applied Biosystems).

2.3. Defining Parasite Lineages

We aligned and edited parasite DNA sequences using Clustal W [49] and Bioedit [50] with the published sequences of other avian malaria parasites registered in GenBank. Lineages differing by one nucleotide were re-sequenced for verification purposes. Many of the sequences obtained differed by as few as one or two nucleotides over the 479 bp examined. Thus, because some avian malaria *Cyt b* lineages with less than 0.5% sequence

divergence may represent different species [51], the same person (JTG) carefully examined each sequence at least twice, discarding those with ambiguous sites (then amplified and sequenced again) to assess the quality of the data. We did not find any individual showing double peaks in the sequences which could indicate the presence of multiple infections [52,53]. Here, we used a threshold of one nucleotide divergence to define separated lineages [54]. All new sequences have been deposited in GenBank (accession numbers EU395835–EU395841).

Although we did not have information on the morphological identity of our lineages, we inferred taxonomic identity by assessing the phylogenetic relatedness of partridge-isolated *Plasmodium* spp. lineages with published sequences from GenBank of morphologically identified parasites (see [55] for a similar procedure) and compiled in the MalAvi database [56]. We estimated the phylogenetic relationships among avian malaria haplotypes using BEAST v.2.5 [57]. The analysis was conducted for 36 lineages of *Plasmodium* and 3 lineages of *Haemoproteus*, together with the 8 *Plasmodium* lineages detected in *A. rufa* (see results). We conducted BEAST analyses using the GTR + I + G model (α = 0.3810; p.inv = 0.312), as selected using jModelTest 2.1.4 [58] under the Akaike Information Criterion (AIC), with estimated base frequencies, relaxed lognormal clock, and two independent MCMC runs of 10 million generations each, sampling every 1000 generations. We checked for convergence using Tracer v. 1.7.1 [59], confirming that ESS values for likelihoods and all parameters were >200. We then combined runs with LogCombiner [60] using 20% burn-in, and generated a maximum clade credibility (MCC) tree in TreeAnnotator [61] from 8002 posterior trees.

Sequence divergence values between *Plasmodium* spp. lineages were analysed using uncorrected P distance.

2.4. Host Mitochondrial Lineages

To differentiate host mtDNA haplotypes within our samples we used sequence variation in the mitochondrial NADH dehydrogenase subunit 2 gene (ND2). We amplified the first part of the ND2 gene using the primers L5216 and H5766 [62]. All samples were sequenced from both ends using the BigDye Terminator Kit, and DNA sequences were determined using an ABI 3100 automated sequencer (Applied Biosystems). Sequence alignment was performed with BioEdit 7.0 [50], together with the sequences of *A. chukar* published in GenBank and *A. magna*, which was used as outgroup. We constructed a neighbour-joining (NJ) tree via PAUP* 4.0 [63], using previously characterized *A. rufa* specimens [32] and published sequences from *A. chukar*. We then assigned each of our *Alectoris* samples to one (*A. rufa*) or the other (*A. chukar*) of the haplogroups.

2.5. Statistical Analysis

Statistical analyses were conducted in Statistica 8.0 [64]. We tested the probability of infection (0 for absence and 1 for presence) in a generalized linear model with logit link function and binomial distribution of errors. First, we investigated the relationship between the infection status (binary variable) and two explanatory variables: the management (game estates with releases vs. game estates without releases) and the sampling period (autumn vs. spring). Second, we performed additional analyses for the two groups of parasites inferred from the phylogenetic analyses (see the Results section). For this, we pooled these parasite lineages with high bootstrap support (>80% posterior probability, Group A and B; Figure 2), categorizing each host sample as either being infected or not infected by each parasite group.

For the subset of infected hosts, we used a log-linear analysis to determine whether the proportion of hosts infected by parasites belonging to Groups A and B was independent of management type and period ($2 \times 2 \times 2$ contingency table; [64]). The Log-Linear analysis is considered an ANOVA-like design for frequency data. Specifically, it is used to test the different factors that are used in a crosstabulation with categorical factors and their interactions for statistical significance [64].

To assess the incidence of partridge restocking in our sample set we compared the occurrence of allochthonous host mtDNA haplotypes (mtDNA corresponding with A. chukar lineages) between autumn and spring in sites with releasing activity by means of a generalized non-linear model (GLZ procedure in Statistica software), and in the single site without releasing activity using a Fisher exact test.

3. Results

Malaria infections were detected in 103 individuals out of 189 red-legged partridges screened, which represented an overall prevalence of 54% (Table 1). All parasite *Cyt b* lineages recorded corresponded to *Plasmodium* spp. (Figure 2), whereas sequences belonging to *Haemoproteus* spp. were absent in our sample set. Eight unique *Plasmodium* lineages were defined according to 43 variable nucleotide sites. There were no insertions or deletions, and thus the nucleotide alignment was unambiguous.

Two of the parasite lineages found in red-legged partridges (SGS1 and GRW11; Figure 2) are considered cosmopolitan in distribution, highly invasive, and have been recorded in more than 140 bird species—principally passerines—in over 43 countries [4,56]. The remaining six lineages corresponded to new parasite lineages, showing divergences between 0.2% and 7.8% with respect to any other *Plasmodium* morphospecies recorded to date in avian hosts. The most common parasite lineage was ARF1 (41% of detected lineages), followed by SGS1 (28%), ARF6 (10.7%), and GRW11 (10.7%). The remaining four lineages (ARF3, ARF5, ARF7 and ARF9) were detected at low rates (less than 5% of infections).

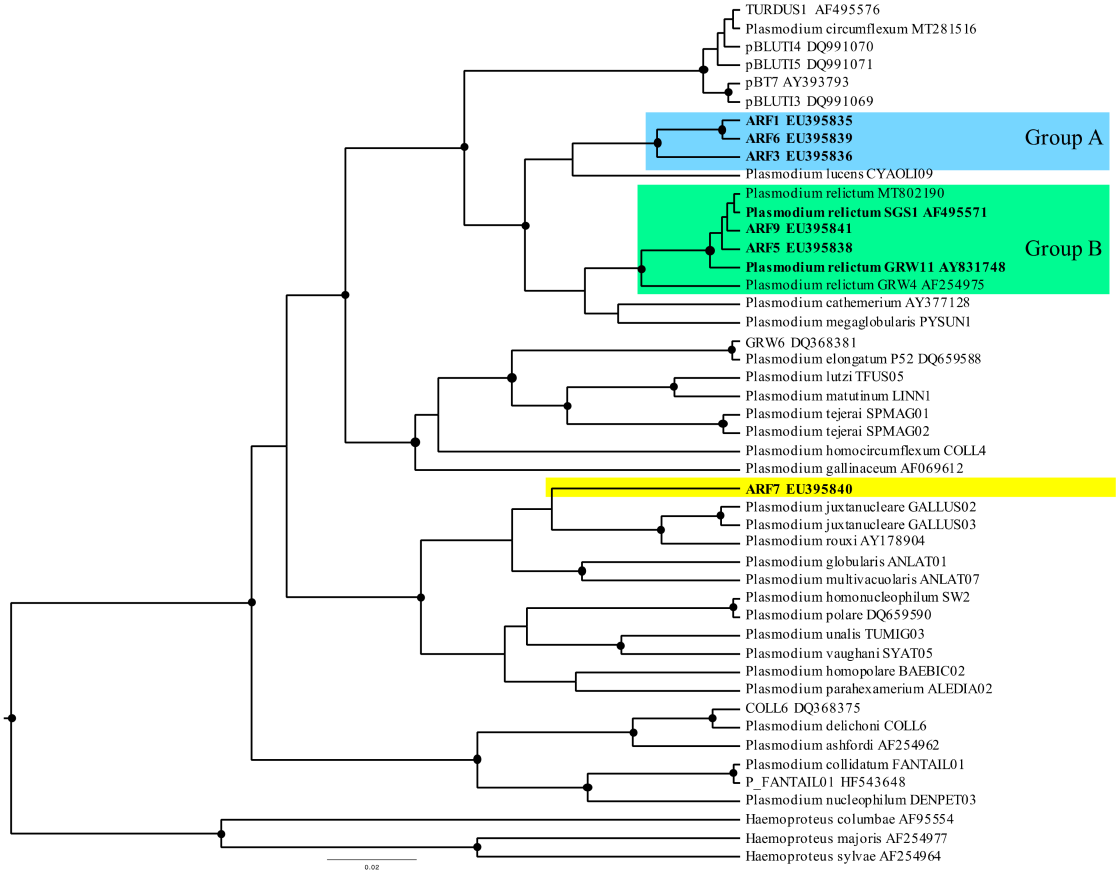

Figure 2. Phylogram of Plasmodium spp. cytochrome b lineages found in *Alectoris rufa* sampled in central Spain. Lineages detected in this study are shown in bold. Principal groups are indicated by colours and correspond to closely related lineages that belong, or likely belong, to the same parasite morphospecies. *Haemoproteus majoris*, *H. columbae* and *H. sylvae* were used as outgroups. Bayesian posterior probabilities above 0.5 are depicted (black dots) at each node. GenBank accession numbers or MalAvi database morphospecies are indicated after lineage names.

3.1. Releasing Activity and Distribution of Host Genotypes

We detected two *A. chukar* and eight *A. rufa* haplotypes in the sample. The alignment of the partridge ND2 sequences yielded a NJ tree in which *A. rufa*, and *A. chukar* haplotypes clustered into two different groups (Figure 3). As predicted, the temporal distribution of partridge haplotypes in sites with releasing activity, but not in sites without releases, showed widespread occurrence of allochthonous mtDNA lineages principally during the hunting season (autumn). In sites with releases, haplotypes belonging to the *A. chukar* clade were significantly higher in autumn (59% of *A. chukar* haplotypes vs. 41% of *A. rufa* clade) than in spring (18% of haplotypes vs. 82% belonging to *A. rufa* clade) ($\chi^2_1 = 4.5$, $p = 0.033$). The occurrence of *A. chukar* haplotypes was similar between both sites with partridge releases ($\chi^2_1 = 0.53$, $p = 0.47$), and the interaction site x period was also non-significant in the model $\chi^2_1 = 0.12$, $p = 0.73$). In contrast, in the game estate without releases (site A), the occurrence of allochthonous mtDNA haplotypes remained seasonally stable (12% *A. chukar* vs. 87% *A. rufa* in autumn, and 8% *A. chukar* vs. 92% *A. rufa* in spring; Fisher exact test, $p = 0.63$). Thus, allochthonous mtDNA haplotypes (i.e., the *A. chukar* clade) appeared more frequently in areas where farm-bred partridges were released for hunting, and principally during the hunting season.

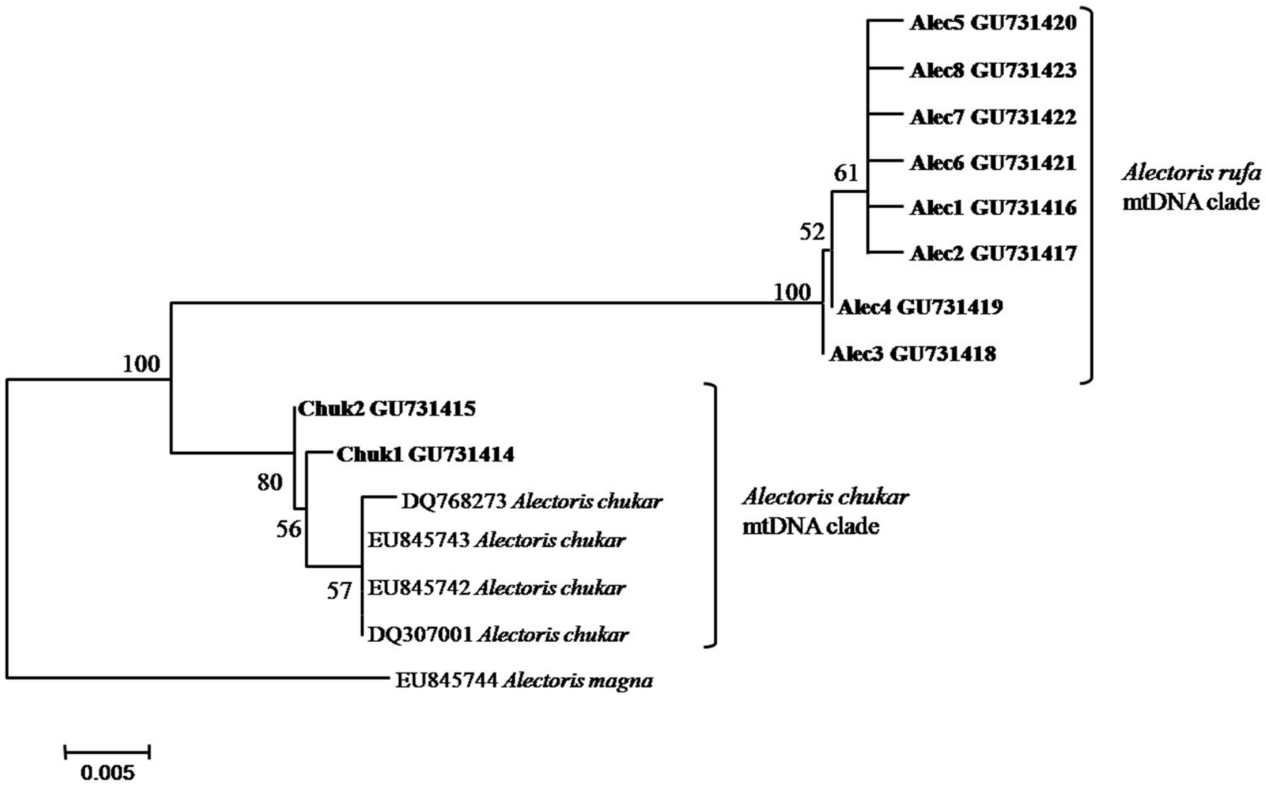

Figure 3. Neighbour-joining tree of mtDNA haplotypes from samples classified morphologically as Alectoris rufa (in bold). The tree was rooted using the Alectoris magna sequence (Genbank accession numbers of sequences are indicated on the tree). Bootstrap support to internal branches (>50; 10, 000 replicates) is indicated by numbers.

3.2. Avian Malaria Lineages of Red-Legged Partridges

The phylogenetic tree (Figure 2) supported the existence of at least three different parasite species among our samples. Three of the lineages (ARF1, ARF3, and ARF6) clustered together into a well-supported clade (>80% posterior probability, Group A; Figure 2), which did not contain sequences of any of the morphospecies included in the analysis. Our isolates SGS1, ARF5, GRW11 and ARF9 did fall together into the *P. relictum* clade (Group B; Figure 2) and we tentatively considered these lineages as belonging to the same morphospecies (*P. relictum*) for further analyses. The genetic distances between

the lineages of Group A ranged from 0.2% to 1.7%, and in Group B from 0.2% to 2.3% (Figure 2). Finally, ARF7 did not match any described morphological species and was placed in the resulting phylogeny in a different clade, thus corresponding to a separate species. Between-group mean genetic distances varied from 2.9% (Group A/Group B) to 16.9% (Group A—*P. tejerai*). Lineage ARF7 exhibited much larger differences with respect to all other lineages, ranging from 5.3% (ARF 7—*P. paraxehamerium*) to 27.8 % (ARF 7—*P. juxtanucleare*). This lineage was excluded from further analyses due to its low occurrence in our populations (Table 1).

3.3. Seasonal Distribution of Parasites and Releasing Activity

We detected red-legged partridges infected by *Plasmodium* spp. in all four sampled sites, and during both sampling periods. The frequency of the eight lineages isolated in red-legged partridges was not randomly distributed among sites (Table 1; Figure 1). Among infected birds, we found large between-population variations in the occurrence of each lineage (Figure 1). Even when considering only lineages present in all the four sampling sites (ARF1 and SGS1), large differences in the relative frequency among different host populations were observed (Figure 1). Tentatively, and considering isolates of the same parasite cluster as belonging to the same species (Figure 2), we observed differences in the probability of being infected in relation to releasing activity and period. Overall, *Plasmodium* spp. prevalence was significantly higher in autumn than in spring (Table 2), with no significant differences between types of management. The interaction of period x management was also non-significant.

Table 2. Generalized linear model (GLZ) for effects of period and management on infection by avian malaria parasites in red-legged partridges.

	Wald $\chi 2$	d.f.	p
All Parasites Pooled			
Period	7.77	1	0.005
Management	0.24	1	0.6
Period x Management	2.76	2	0.1
Parasites of Group A			
Period	0.13	1	0.71
Management	12.12	1	0.0004
Period x Management	0.04	2	0.95
Parasites of Group B			
Period	4.51		0.033
Management	9.04	1	0.0026
Period x Management	2.57		0.11

At the parasite-group level, the prevalence showed a significant but opposite effect, with a higher prevalence in sites with releases than sites without releases for Group B, whereas Group A exhibited the inverse, with a higher prevalence in non-releasing than in releasing sites (Table 2). Indeed, we found a season effect on Group B prevalence, with a higher prevalence during autumn than during spring. No effect of period was found on the prevalence of Group A, and neither was the interaction period x management significant. The distribution of parasite lineages among infected hosts gave similar results. The Log-Linear analysis indicated that the best model for parasite distribution included all the second-order interactions, with no single or three-way interaction (all $p > 0.05$). The management-by-period interaction ($\chi^2_4 = 19.38$, $p = 0.006$; Figure 4) indicates that parasites were more frequent in autumn than in spring in sites with releasing activity, whereas no between-period differences were found at game estates without releases. Parasites belonging to Group A were more frequent in sites without releases than in sites with releases (Figure 4) whereas the opposite was found for Group B (management-by-group

interaction: $\chi^2_4 = 11.97$, $p = 0.017$). Furthermore, the frequency of parasites in Group A did not vary significantly between spring and autumn. In contrast, Group B lineages were much more frequent in autumn than in spring in sites with releases, but not in sites without releases (group-by-period interaction: $\chi^2_4 = 23.78$, $p = 0.00009$; Figure 4).

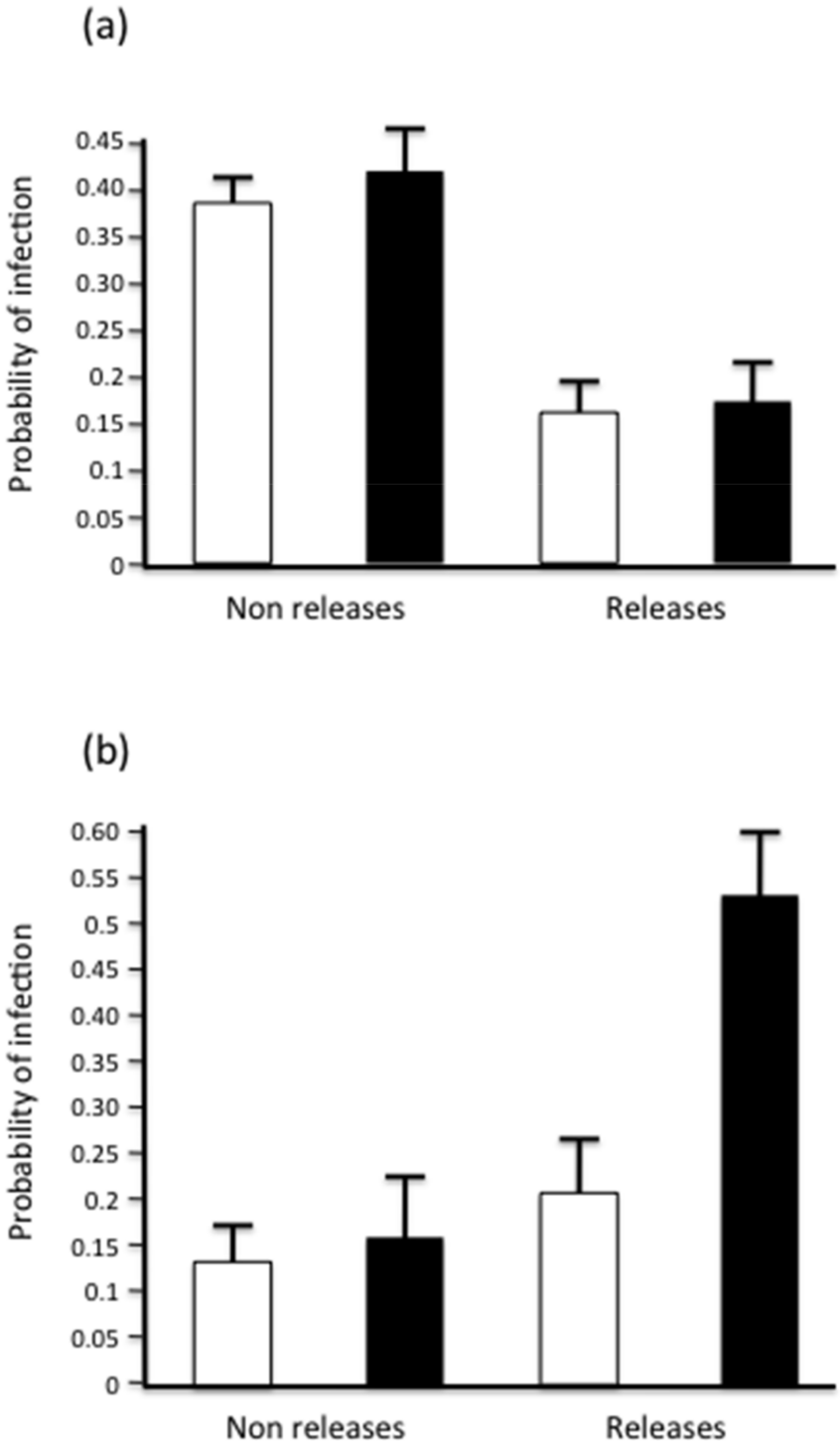

Figure 4. Probability of infection in red-legged partridges in game estates with releases and without releases with avian malaria parasites belonging to (**a**) Group A or (**b**) Group B of parasites, according to Figure 2. We show the weighted marginal means (±S.E.) during both spring (white bars) and autumn (black bars) sampling periods.

The parasites detected here can be further split into relation-to-host haplotypes: lineages infecting the *A. chukar* clade and lineages infecting the *A. rufa* clade. Six out of the eight parasite lineages were present in both host haplotypes, while two parasite lineages (ARF3 and ARF7) were absent from *A. chukar* hosts. Overall, parasites belonging to Group A were more frequent in *A. rufa* hosts (57.5% of all infections in these hosts) than in *A. chukar* hosts (34.8%), while parasites of Group B were more frequent in *A. Chukar* (65%) than in *A. rufa* hosts (40%).

4. Discussion

As humans continuously alter landscapes and ecological communities, the knowledge of how parasites and their hosts respond to these changes and the implications and consequences for the transmission and distribution of infectious agents are of paramount importance [10–12]. We found a quite high prevalence of avian malaria parasites in red-legged partridges in central Spain. The results presented here revealed malaria prevalences that are among the highest found in bird populations, particularly for *Plasmodium* [4,65–68], including studies on species from comparable habitats in Spain [69]. We also found notable differences in the spatial and temporal distribution of parasites at a local scale. Our results show that parasite species can differ in their distribution and temporal abundance with regard to game management type (sites with red-legged partridge releases and sites without releases). This distribution of parasites may potentially be caused by the effect of large-scale and widespread human activity (releases into the wild of farm-reared birds), as recently reported for the highly invasive lineage SGS1 in passerine birds of the USA [4].

Despite the socioeconomic and conservation relevance of red-legged partridges, there is still very limited knowledge on some pathogens that can threaten their long-term conservation [30,70]. In particular, very few studies are available on avian malaria in this species (a recent review of partridge diseases can be found in [70]) and we were unable to find similar studies based on DNA analyses for this group of parasites. Therefore, we can only compare our results with studies based on the analyses of blood smears. [71] reported an overall prevalence of *Plasmodium relictum* in wild partridge populations from western Spain of between 3.2% and 16.6%, while [72] examined farm-reared partridges from central Spain and found that *Haemoproteus* spp. occurred in 10% of birds. Apart from the few studies carried out in Spain, the occurrence of *Haemoproteus* sp. in *A. rufa* was reported for the first time recently in NW Italy [73].

The high prevalence of *Plasmodium* spp. in our study as compared with previous works may be related to spatial heterogeneity and/or temporal shifts in infestation rates [74], or to differences in the detection power of research protocols (blood smears vs. molecular identification, [48]). In general, the absence or low prevalence of hematozoan recorded in many host species inhabiting open and arid environments has been commonly attributed to a reduced transmission rate of parasites due to the scarcity of suitable vectors in those habitats (review in [75]). However, our findings suggest that the abundance of suitable vectors (Culicidae mosquitoes; [69] should not be a limiting factor for the existence and dispersion of malaria parasites in agricultural habitats, at least in our study area (see also [67] for the Mediterranean island of Sardinia). Higher prevalences of these parasites in birds have been found in areas with elevated summer NDVI (a widely used index of vegetal productivity) at the continental level [65], but our study area has a semi-arid climate with very dry summers and consequently is expected to have a low summer NDVI. Future research should measure the density and the parasites that the (infected) vectors carry to confirm this hypothesis.

The absence of *Haemoproteus* infections in our sample set may be due to the primer sequences being more similar to *Plasmodium* than to *Haemoproteus*, or because *Plasmodium* reaches higher parasitemias than *Haemoproteus* [53]. This is expected if the PCR assays are developed using conserved *Plasmodium* spp. sequences, and hence often preferentially amplify the DNA of parasites of this genus (see [76] for the shortcomings of different PCR assays)

At the local scale of our study, there was compelling evidence of a relationship between releasing activity and parasite infection. The seasonal distribution of parasites in game estates where partridge restocking had occurred differed from that of game estates without releases. In the two sites with releasing activity, parasite prevalence was higher in autumn (when hunters restocked partridge populations) than in spring (when most of the released partridges had been hunted; [77]), while the high frequency of allochthonous host mtDNA haplotypes among the autumn samples confirmed the captive breeding origin of hunted birds [32]. This is consistent with the findings of [30,78], who suggested that partridge releases promote parasite opportunities for infecting wild populations and generating subsequent host-to-host transmission (see also [79]). Most of the released partridges do not survive the first hunting season [39–41], as suggested by the sharp reduction observed in the abundance of hybrids (*A. chukar* haplotypes) after the hunting season in sites with releases, but the overall host-to-host transmission rate would depend on both the survival rate of the released birds and the number of partridges released yearly. In the study area over the years, the mean number of red-legs released in traditional shooting partridge estates was about 8465 birds per season, although in intensive estates, the mean was 21,408 birds, with some estates releasing up to 90,000 birds [80]. Consequently, although the number of survivors was usually very low, the total transmission rate to wild birds could be not negligible. We cannot definitively discard the potential effect of sampling bias between periods, due to the noticeable reticence of hunting managers in allowing sampling from farmed stocks previous to releases. Since we only have data on hunter-harvested birds, one could argue that this handicap may affect the results obtained, as infected birds might be more susceptible to being hunted than uninfected birds. However, the hunting modality used in our study area where long beater rows, often with dogs running ahead, drive the partridges towards another row of shooters, do not necessarily imply selection against weak, infected or low-condition individuals. Rather, the probability of killing a partridge is more related to the ability of the shooter than to the condition of birds.

On the other hand, the parasite assemblage included the presence of two previously described lineages of *Plasmodium* (SGS1 and GRW11), which emphasizes the general idiosyncrasy of these parasite lineages, both geographically and taxonomically (i.e., regarding the broad range of hosts infected [4,54,56,81–83]). Of the eight parasite lineages isolated in partridges, six *Plasmodium* spp. have not been previously detected in avian hosts. This may reflect the limited sampling of avian hosts at our study sites or the specificity for hosts of these lineages.

The prevalence of 'generalist' parasites (Group B) was higher in the two sites with releases than in sites without them, and principally during autumn. In contrast, the prevalence of 'partridge-specific' parasites belonging to Group A (Figure 2) was higher in sites without releases than in sites with releases, and was found in similar proportions in spring and autumn samples. It is plausible that the potential for transmission at different points of the year may differ between different parasite species, although the knowledge on the seasonal variation of prevalences of these parasites is quite limited [84]. For example, the seasonal pattern of prevalence in partridges is clearly different from the two *Plasmodium* species infecting blue tits (*Cyanistes caeruleus*) in UK [85]. The general seasonal pattern seems to be that the prevalence of avian malaria parasites increases during the breeding season in temperate wild bird populations, reaching maximal values during summer and then declining through autumn [84,86], a pattern corroborated by the *P. relictum* data [85]. However, recent work with house sparrows (*Passer domesticus*) in Spain reported a double peak pattern of *Plasmodium* prevalence in spring and autumn [87]. Overall, the most likely explanation for the seasonal pattern we found in partridges is that parasites belonging to Group B (that included generalist lineages) correspond with the parasites harbored by farm-reared partridges that were released into the wild in autumn. Indeed, the distribution of parasites within the two host groups (allochthonous vs. autochthonous mtDNA haplotypes) further supports this hypothesis.

The acquisition of generalist lineages from infected (local) hosts is likely to occur at game bird facilities from local avifauna. Partridges born and bred on farms were often infected by avian malaria before the time of release, especially via host-to-host transmission from passerines that come to farms in high densities to exploit partridge feeders and drinkers (authors, unpublished data). Thus, relocation of farming stocks in distant locations may lead to local differences in parasite composition and specific abundance. An alternative explanation for the higher prevalence of this group of parasites in autumn than in spring in sites with releases is the greater susceptibility of released birds to local parasite lineages once they are in the field. However, when releases occur in the study area (late summer/early autumn) the abundance of suitable vectors in the field should be low (mean temperatures in October averaged 16 °C and minimum temperatures averaged 11 °C). Consequently, at that time, it is more likely that the parasites sampled already existed in the hosts rather than resulting from new infections after releases. The strong decline of Group B prevalence from autumn to spring in game estates with releases may be explained by the disappearance of released birds due to hunting, or to the high natural mortality typical of traditional restocking [40,77].

5. Conclusions

Further work is needed to determine if captive game breeding facilities could be considered as reservoir hosts and foci of malaria parasites, which can potentially spill out to attack new hosts of distant areas via introductions or translocations of infected animals, as the available information is very scarce. Gamebird releasing is a broadly accepted and very common activity in most countries, and the potential effects of parasite introduction into new environments should not be discarded—on the contrary, it should be increasingly considered in evolutionary ecology and conservation biology. A human-induced increase in prevalence or diversification of the parasite community would induce new immune challenges in wild bird populations [88], maybe reducing survival or jeopardizing other host life history traits in new hosts [25,89]. Evidence concerning the effects of blood parasites in wild non-passerine birds is however limited, and in red-legged partridges, completely absent. In light of our results, the potential pathogenic effects of avian malaria parasites in wild partridge populations may be added to the other negative effects of releases for wild host populations [42], such as the spreading of intestinal parasites [28,29], genetic introgression [32,34,90], or over-hunting [77,91]. Given the potential of partridge releases to introduce new avian malaria parasites into wild populations, studies on the pathologic effects of these parasites are urgently needed. Tens of millions of game birds are released yearly in Europe [92], but the use of captive stocks for shooting may be harmful for wild populations of target species and may also cause serious side effects on many non-target sympatric avian species [93].

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