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No relationship between individual genetic diversity and prevalence of avian malaria in a migratory kestrel

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Abstract

Insight into the genetic basis of malaria resistance is crucial for understanding the consequences of this parasite group on animal populations. Here, we analyse the relationship between genotypic variation at 11 highly variable microsatellite loci and prevalence of three different lineages of avian malaria, two *Plasmodium* (RTSR1, LK6) and one *Haemoproteus* (LK2), in a wild population of the endangered lesser kestrel (*Falco naumanni*). Although we used a large sample size (584 typed individuals), we did not find any significant association between the prevalence of the studied parasite lineages and individual genetic diversity. Although our data set is large, the 11 neutral markers typed may have had low power to detect such association, in part because of the low parasite prevalence observed (less than 5% of infected birds). However, the fact that we have detected previous correlations between genetic diversity and other traits (ectoparasitism risk, fecundity) in the study population using the same panel of neutral markers and lower sample sizes suggests that other factors could underlie the absence of such a similar correlation with avian malaria. Differences in the genetics of the studied traits and in their particular basis of inbreeding depression (dominance vs. overdominance) may have led to malaria prevalence, but not other traits, being uncoupled with individual genetic diversity. Also, we cannot discard the possibility that the absence of association was a consequence of a low pathogenic effect of these particular malaria lineages on our lesser kestrel population, and thus we should not expect the evolution of genetic resistance against these parasites.

Keywords: *Falco naumanni*, *Haemoproteus*, heterozygosity, lesser kestrel, microsatellites, *Plasmodium*

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Introduction

Understanding the role of individual genetic diversity on risk of parasitism is a central topic in conservation genetics especially at a time when several novel infectious diseases are emerging in natural populations and human-induced habitat loss is causing important reductions of genetic diversity in many species worldwide (O'Brien & Evermann 1988; Acevedo-Whitehouse *et al*. 2003; Altizer *et al*. 2003). Avian malaria parasites are responsible of widespread declines in Hawaiian native forest birds (Van Riper *et al*. 1986) and several negative effects, including increased mortality and reduced reproductive outputs, have been described in several bird species (Atkinson *et al*. 2000; Dawson & Bortolotti 2000; Merino *et al*. 2000; Sol *et al*. 2003). Thus, these parasites are likely to be a strong selective agent for the evolution of host defences and have probably shaped host genomes through selection of several protective genetic traits (Westerdahl *et al*. 2005; Bonneaud *et al*. 2006). Some studies have linked genetic resistance to specific alleles (e.g. Westerdahl *et al*. 2005; Bonneaud *et al*. 2006), and other studies have shown that higher heterozygosity at certain loci increases individual ability to cope with a broader range of malarial infections (e.g. McGuire *et al*. 1994; Westerdahl *et al*. 2005). Further, several other parts of the genome not directly involved in immune defence are also likely to modulate resistance to malaria and other parasites, including genes affecting physiological processes and cellular homeostasis that are known to be sensitive to reduced genome-wide heterozygosity (Pedersen *et al*. 2005; Luong

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et al. 2007). Thus, it may be that the risk of acquiring blood parasites is also associated with gene polymorphisms at many other parts of the genome not directly involved with immune response. However, in spite of the important implications for both ecology and conservation research, few studies have evaluated the role of overall genetic diversity on the risk of acquiring malaria parasites and the available information on the topic is contradictory (MacDougall-Shackleton *et al*. 2005; Westerdahl *et al*. 2005).

The lesser kestrel (*Falco naumanni*), a small size colonial bird of prey, is an interesting model system for exploring the association between individual genetic diversity and susceptibility to diseases (Ortego *et al*. 2007a). First, this species suffered a large population decline in the middle of the 20th century, with an estimated loss of around 95% of the breeding population in the Iberian Peninsula in only 20 years. This may have reduced mean population heterozygosity and increased the expression of the genetic load, potentially magnifying the consequences of reduced individual genetic diversity on parasite susceptibility (Pearman & Garner 2005; Ortego *et al*. 2007a). Second, the lesser kestrel is a long-distance migrant so that it is likely to be exposed to avian malaria parasites of both Europe and Africa (Waldenstrom *et al*. 2002; Ortego *et al*. 2007b). Thus, this species probably shows an increased risk of encounter and being infected with two distinct parasite faunas and it may suffer a higher selective pressure from pathogens in comparison with nonmigratory species (Møller & Erritzoe 2001; Waldenström *et al*. 2002). Finally, the lesser kestrel is a colonial breeder and this may enhance the transmission rate of parasites in their breeding grounds, making this species potentially more sensitive to disease outbreaks (Tella 2002).

Six different avian malaria lineages have been described parasitizing lesser kestrels, with two *Plasmodium* (RTSR1, LK6) and one *Haemoproteus* (LK2) lineages being the most common in our study area from Central Spain (Ortego *et al*. 2007b). The mean prevalence of these three lineages in the study population is 3.4% for RTSR1, 4.6% for LK6 and 4.1% for LK2 and no mixed infections have been detected (Ortego *et al*. 2007b). According to the obtained sequence divergences, these lineages seem to correspond to three well-differentiated species (Ortego *et al*. 2007b) and so they may have evolved different adaptations and shaped host genomes differentially (Bensch *et al*. 2004). In the present study, we used blood parasite data reported in a previous study (Ortego *et al*. 2007b) to investigate the association between individual genetic diversity and prevalence of avian malaria in lesser kestrels. For this purpose, 584 birds previously screened for malaria parasites using a highly efficient polymerase chain reaction (PCR) approach were also typed at 11 highly variable microsatellite loci that allowed us to estimate individual genetic diversity.

Materials and methods

Study population and field procedures

The study was conducted in La Mancha, central Spain (600–800 m above sea level), in an area covering approximately 1000 km^2 (see Ortego *et al*. 2007c for a detailed description). We studied 30 lesser kestrel colonies clustered in two subpopulations separated by 30 km: 'Villacañas' subpopulation (39°30′N, 3°20′W; 24 colonies) and 'Consuegra' subpopulation (39°35′N, 3°40′W; six colonies). However, in spite of the low exchange of individuals between both subpopulations, a preliminary analysis showed an absence of genetic structure (Ortego *et al*. 2007c).

Kestrels normally arrive to the study area from their winter quarters in Africa in mid-February or the beginning of March, depending on the year. During the 2001–2006 breeding seasons, adult lesser kestrels were trapped with a noose carpet or by hand during incubation, measured and individually marked with metallic and coloured plastic rings for further identification. Blood samples (100 μL) were obtained by venipuncture of the brachial vein and preserved in ~1200 μL ethanol 96% at –20 °C. We used pectoral thickness as an estimator of body condition (Aparicio 1997; Aparicio & Cordero 2001). This trait has been used in previous studies as a measure of body condition in several bird species (Bolton *et al*. 1991; Newton 1993), and has been considered a more reliable measure of condition than residuals of body mass on tarsus length (Gosler & Harper 2000). Moreover, it is easy to measure accurately on live birds using a portable ultrasonic metre, in this case a Krautkrämer USM22F (accuracy 0.1 mm), especially designed to measure animal tissues. We knew the exact age of approximately one-third of individuals that were ringed as fledglings. For all other birds, we considered that individuals captured for the first time were in their first year if they presented yearling plumage or in their second year if they presented adult plumage (e.g. Aparicio & Cordero 2001; Foerster *et al*. 2003; Ortego *et al*. 2007a, c). We manipulated and banded lesser kestrels under licence from the Spanish institutional authorities (Environmental Agency of Junta de Comunidades de Castilla-La Mancha and the Ringing Office of the Ministry of Environment), and we followed general ethical guidelines for animal welfare and nature conservation.

Microsatellite genotyping and estimates of genetic diversity

We quantified multilocus heterozygosity in 584 lesser kestrels across 11 highly polymorphic microsatellite markers: Fp5, Fp13, Fp31, Fp46–1, Fp79–4, Fp86–2, Fp89 (Nesje *et al*. 2000), Fu1, Fu2 (J. H. Wetton, unpublished), Fn1–11, and Fn2–14 (Ortego *et al*. 2007d). All individuals were

genotyped at all these 11 microsatellite markers. We used QIAamp DNA Blood Mini Kits (QIAGEN) to extract and purify genomic DNA from the blood samples. Approximately 5 ng of template DNA was amplified in 10-μL reaction volumes containing 1× reaction buffer (67 mM Tris-HCL, pH 8.3, 16 mM $(NH_4)_2SO_4$, 0.01% Tween-20, EcoStart Reaction Buffer, Ecogen), 2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.15 μM of each dye-labelled primer (FAM, HEX or NED) and 0.1 U of *Taq* DNA EcoStart Polymerase (Ecogen). All reactions were carried out on a Mastercycler EpgradientS (Eppendorf) thermal cycler. The PCR programme used was 9 min denaturing at 95 °C followed by 30 cycles of 30 s at 94 °C, 45 s at the annealing temperature and 45 s at 72 °C, ending with a 5-min final elongation stage at 72 °C. Amplification products were electrophoresed using an ABI 310 Genetic Analyser (Applied Biosystems) and genotypes were scored using GENESCAN 3.7 (Applied Biosystems).

In the present study, we use homozygosity by loci (*HL*) to estimate individual genetic diversity, a microsatellite-derived measure that improves heterozygosity estimates in open populations by weighting the contribution of each locus to the homozygosity value according to its allelic variability (Aparicio *et al*. 2006; Ortego *et al*. 2007a, c). *HL* was estimated using CERNICALIN, an Excel spreadsheet available on request.

Screening for avian malarial infections

Each bird was screened for malaria infection using a highly efficient nested PCR protocol that amplifies a 524-bp fragment (including primers) of the mitochondrial cytochrome *b* gene of both *Plasmodium* and *Haemoproteous* parasites (Waldenstrom *et al*. 2004). This method consists in two rounds, an initial 20 cycles of PCR using the primers HAEMNF and HAEMNR2 and a final 35 cycles of PCR using the internally nested primers HAEMF and HAEMR2 (Waldenstrom *et al*. 2004). For the second PCR, 1 μL of the PCR product from the initial PCR was used as template. All PCRs were performed in 25 μL volumes, and we routinely used positive (i.e. DNA from individuals with known malarial infections) and negative controls (i.e. samples with double-distilled water instead of genomic DNA as template) to ascertain that the outcome of each PCR run was not affected by contamination (Waldenstrom *et al*. 2004). The PCR programme, thermal profile, and reagent proportions were as described by Waldenstrom *et al*. (2004), with the exception of using a 9-min denaturing at 95 °C rather than 3 min at 94 °C because we used a hot-start polymerase (EcoStart, Ecogen). Reagents were the same used for microsatellite amplifications (see above). Amplification of the 11 microsatellite loci described above was used as a control for DNA quality. These amplifications were successful in all cases. Further, negative infections were confirmed by repeated PCR. In a previous study including a subset of the samples used here ($n = 288$), we found that all infections detected by ocular screening of blood smears gave positive PCR amplifications while several PCR detected infections could not be determined using ocular examinations (Ortego *et al*. 2007b). As found in several studies, this indicates that the nested PCR protocol is much more sensitive than the traditional microscopy-based examinations of blood smears (Waldenstrom *et al*. 2004). Positive or negative second round PCR products (i.e. birds having or no gametocytes or merozoites in their blood stream) were scored by electrophoresis on 2% agarose gels stained with ethidium bromide and determining the presence/absence of a band of the expected size under UV light. PCR products from positive samples were purified using NucleoSpin Extract II (MACHEREY-NAGEL) kits and bidirectionally sequenced on an ABI 310 Genetic Analyser (Applied Biosystems). Sequences were edited and aligned using the program BIOEDIT (Hall 1999).

Statistical analyses

Tests for deviation from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium between markers were calculated following Weir (1996). We analysed the relationship between *HL* and malaria prevalence (584 individuals) using generalized linear mixed models (GLMMs) implemented with the GLIMIX macro of SAS (SAS Institute 2004). GLMMs allow analyses of data where the response variable is determined by both random and fixed effects. We analysed total prevalence of avian malaria parasites and prevalence of the three most common lineages (RTSR1, LK2, LK6; Ortego *et al*. 2007b) isolated from lesser kestrels breeding in the study population. GLMMs were constructed by fitting *HL* as explanatory variable together with nongenetic terms (covariates: pectoral thickness, age, colony size, day of capture; fixed factors: locality, year, sex) that could potentially influence malaria parasite prevalence (e.g. Dawson & Bortolotti 1999; Dawson & Bortolotti 2000; Tella 2002). No pair of these independent variables was strongly correlated (all $r < 0.35$) so we initially included all of them in our analyses (Green 1979). The identities of colonies and cohort were included as random effects to control for the potential nonindependence of parasite prevalence within colonies and cohorts, in the manner of a randomized complete block design to avoid pseudoreplication (Krackow & Tkadlec 2001). Finally, we assessed for the possible effects of single-locus heterozygosity on malaria prevalence. For this purpose, we re-analysed our data including into the models heterozygosity at each marker as a binary variable (e.g. Lieutenant-Gosselin & Bernatchez 2006).

Initially, each GLMM was constructed with all explanatory terms fitted, including first-order interactions

Table 1 GLMMs (binomial error and logit link function) for total prevalence (all lineages pooled) and prevalence of the three most common malaria lineages (RTSR1, *Plasmodium* sp., LK6, *Plasmodium* sp., LK2, *Haemoproteus* sp.) parasitizing lesser kestrels in relation to homozygosity by loci (*HL*) and nongenetic terms (covariates: pectoral thickness, age, colony size, day of capture; fixed factors: locality, year, sex)

	d.f.	*F*	*P*
(a) Total prevalence			
HL	1, 582	0.31	0.577
Pectoral thickness	1, 582	0.01	0.932
Age	1, 582	1.32	0.251
Colony size	1, 582	0.68	0.410
Day of capture	1, 582	0.42	0.518
Locality	1, 582	0.00	0.994
Year	5, 578	0.73	0.601
Sex	1, 582	0.99	0.321
HL × locality	1, 580	1.01	0.316
HL × year	5, 572	0.42	0.835
HL × sex	1, 580	0.10	0.754
Covariance parameter estimates:	*Z*	*P*	
Colony identity	—	—	
Cohort	—	—	
(b) Prevalence of RTSR1			
HL	1, 582	0.00	0.961
Pectoral thickness	1, 582	0.18	0.675
Age	1, 582	0.54	0.462
Colony size	1, 582	0.43	0.510
Day of capture	1, 582	1.67	0.197
Locality	1, 582	2.20	0.139
Year	5, 578	0.95	0.448
Sex	1, 582	0.05	0.825
HL × locality	1, 580	0.23	0.632
HL × year	5, 572	0.40	0.848
HL × sex	1, 580	0.09	0.762
Covariance parameter estimates:	*Z*	*P*	
Colony identity	—	—	
Cohort	—	—	
(c) Prevalence of LK6			
HL	1, 582	0.24	0.627
Pectoral thickness	1, 582	0.93	0.335
Age	1, 582	0.73	0.394
Colony size	1, 582	0.06	0.807
Day of capture	1, 582	0.60	0.437
Locality	1, 582	2.93	0.087
Year	5, 578	0.14	0.984
Sex	1, 582	0.50	0.478
HL × locality	1, 580	0.50	0.479
HL × year	5, 572	0.32	0.904
HL × sex	1, 580	0.08	0.775
Covariance parameter estimates:	*Z*	*P*	
Colony identity	—	—	
Cohort	—	—	
(d) Prevalence of LK2			
HL	1, 582	0.75	0.388
Pectoral thickness	1, 582	1.35	0.245
Age	1, 582	0.83	0.362
Colony size	1, 582	0.95	0.329
Day of capture	1, 582	3.38	0.066
Locality	1, 582	0.60	0.437
Year	5, 578	1.78	0.115
Sex	1, 582	0.03	0.873
HL × locality	1, 580	0.09	0.761
HL × year	5, 572	0.81	0.545
HL × sex	1, 580	0.18	0.669
Covariance parameter estimates:	*Z*	*P*	
Colony identity	—	—	
Cohort	—	—	

and quadratic effects to account for potential nonlinear relationships. Final models were selected following a backward procedure, by progressively eliminating nonsignificant variables. The significance of the remaining variables was tested again until no additional variable reached significance. The result is the minimal and most adequate model for explaining the variability in the response variable, where only the significant explanatory variables are retained. All tests were performed using the residual degrees of freedom (SAS Institute 2004). Hypotheses were tested using *F*-statistics and all *P* values refer to two-tailed tests.

Results

The mean number of alleles per locus was 25.7, and ranged from three to 128. After applying sequential Bonferroni corrections to compensate for multiple statistical tests, four loci deviated significantly from HWE (Fp13, Fp86–2, Fu2, Fn2–14). There was no evidence of genotypic linkage disequilibrium at any pair of loci and subpopulation (all $P > 0.05$). *HL* was not correlated with total prevalence or prevalence of any particular lineage (Table 1; Fig. 1). The interactions between *HL* and year, sex, and locality were all not significant, indicating that the absence of association between genetic diversity and prevalence of malaria was consistent between years, sexes and localities (Table 1). We repeated the analyses excluding from *HL* calculation the four loci that departed significantly from HWE, and we found qualitatively equal results (total prevalence: $F_{(1,582)} = 1.73$, $P = 0.189$; RTSR1: $F_{(1,582)} = 0.07$, $P = 0.796$; LK6:$_{(1, 582)} = 0.27$, $P = 0.603$; LK2: $F_{(1,582)} = 0.57$, $P = 0.451$). Colony size, age, pectoral thickness, year, and sex did not significantly influence parasite prevalence either (Table 1). Day of capture tended to be negatively associated with prevalence of LK2 (estimate ± SE = 0.0008 ± 0.0004; $P = 0.066$; Table 1d), suggesting a reduction of infection intensity over the summer (Waldenstrom *et al*. 2004; Westerdahl *et al*. 2005). However, day of capture had no effect on prevalence of the other two lineages or total prevalence (Table 1). Only the prevalence of LK6 showed a marginally significant difference between localities, being higher in Villacañas than in Consuegra ($P = 0.087$; Table 2c). Such spatial variation in prevalence of LK6 may suggest that this lineage could be transmitted in the study area. In Villacañas, there are several lagoons that may enhance the proliferation of potential vectors for haemoparasite transmission in comparison with Consuegra (Ortego & Espada 2007). Quadratic terms and other interactions between independent variables were not significant in any analysis ($P > 0.1$ in all cases). After applying sequential Bonferroni corrections to compensate for multiple statistical tests, we found no significant effect of any single locus on prevalence of avian malaria (Table 2).

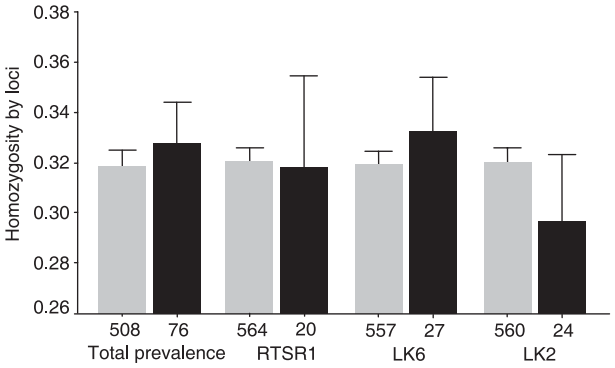

Fig. 1 Homozygosity by loci (mean ± 1 SE) for uninfected (grey bars) and infected (black bars) lesser kestrels with avian malaria. Data are presented pooling all malaria lineages (total prevalence) and for each individual lineage of *Plasmodium* (RSRS1, LK6) and *Haemoproteus* (LK2). Figures under bars indicate sample size in each group.

Table 2 Test for effects of single-locus heterozygosity on total prevalence (all lineages pooled) and prevalence of the three most common malaria lineages (RTSR1, *Plasmodium* sp., LK6, *Plasmodium* sp., LK2, *Haemoproteus* sp.) parasitizing lesser kestrels. Table shows *P* values. No single-locus effect remained significant after applying sequential Bonferroni corrections

Locus	Total prevalence	RTSR1	LK6	LK2
Fp5	0.909	0.246	0.643	0.038
Fp13	0.822	0.876	0.902	0.161
Fp31	0.136	0.057	0.485	0.904
Fp46–1	0.160	0.641	0.202	0.862
Fp79–4	0.611	0.439	0.836	0.722
Fp86–2	0.158	0.530	0.726	0.221
Fp89	0.248	0.154	0.625	0.357
Fu1	0.139	0.061	0.657	0.078
Fu2	0.777	0.349	0.774	0.257
Fn1–11	0.928	0.405	0.591	0.791
Fn2–14	0.749	0.957	0.972	0.385

Discussion

Here, we analyse the relationship between individual genetic diversity at 11 highly variable microsatellite loci and prevalence of avian malaria in 584 lesser kestrels breeding in La Mancha, Central Spain. Although we used a large sample size and a typical data set of 11 neutral markers, we found no significant association between individual genetic variation and prevalence of any of the three most common lineages of avian malaria parasitizing adult lesser kestrels in our study population. Similarly, Westerdahl *et al*. (2005) also reported no relationship between individual heterozygosity at 18 microsatellite loci and prevalence of avian malaria in a sample of 340 great reed warblers (*Acrocephalus arundinaceus*). In contrast, MacDougall-Shackleton *et al*. (2005) found a negative association between *Haemoproteus* sp. prevalence and individual heterozygosity in a wild population of mountain white-crowned sparrows (*Zonotrichia leucophrys*) although they only typed 48 individuals at eight neutral markers.

Recent theoretical and empirical studies have shown that the small number of neutral markers (eight to 20) generally used to estimate genome-wide heterozygosity is only expected to correlate weakly with inbreeding coefficients even when the number of typed individuals is large (Balloux *et al*. 2004; Slate *et al*. 2004). Thus, one possible explanation for the absence of association between microsatellite variation and prevalence of malaria in our study population is that the 11 neutral markers employed may have provided a poor estimate of heterozygosity across the whole genome and we could have lacked statistical power enough to detect such correlation in spite of our extensive data set. However, several intrinsic characteristics of the study population, such as the possibility of admixture of genetically differentiated individuals during the recent demographic expansion of the study population (J. Ortego, unpublished) and the philopatric behaviour of this species, suggest that the study population may show enough inbreeding variance to be reflected in neutral markers (Ortego *et al*. 2007a, c). Further, we have previously detected correlations between multilocus heterozygosity and both clutch size (Ortego *et al*. 2007c) and prevalence of feather lice (Ortego *et al*. 2007a) in the studied lesser kestrel population using the same panel of microsatellite loci and a smaller number of typed individuals. In these two previous studies, the obtained correlations were not fully explained by any particular locus, suggesting that genome-wide heterozygosity may be involved and local effects (i.e. the possibility of linkage disequilibrium between markers and functional genes; David 1998; Hansson *et al*. 2001; Hansson & Westerberg 2002) were not the underlying mechanism. Thus, it is not likely that the panel of markers used lie close enough to functional genes experiencing balancing selection that influence clutch size and lice prevalence but not the risk of acquiring malaria infections. Further, there was also no evidence for local effects of the analysed microsatellite markers on prevalence of any of the studied lineages of avian malaria. This raises the question, why is individual genetic diversity uncoupled with malaria prevalence but correlated with other traits in the studied lesser kestrel population? This may occur for several reasons.

If we assume that malaria infection has fitness consequences in lesser kestrels, as found in other bird species (Oppliger *et al*. 1997; Dawson & Bortolotti 2000; Merino *et al*. 2000; Sol *et al*. 2003), one plausible explanation for the results invokes a possible reduction of genetic diversity that occurred after the generalized population decline experienced by lesser kestrels in the middle of the 20th

century. This population reduction could have increased the expression of the genetic load for malaria resistance, purging deleterious recessive alleles and thus uncoupling the predicted relationship between infection resistance and heterozygosity (Dudash *et al*. 1997; Wiehn *et al*. 2002; Côte *et al*. 2005). Feather lice are suspected to show much lower levels of pathogenicity than malaria parasites, whereas clutch size, despite being closely linked to fitness (Ortego *et al*. 2007c), may be submitted to weaker selection than resistance to avian malaria, which has been found to reduce both fecundity and adult survival in several bird species (Oppliger *et al*. 1997; Dawson & Bortolotti 2000; Merino *et al*. 2000; Sol *et al*. 2003). Thus, the reduction of the genetic load could have been more efficient for malaria resistance and alleles with purely detrimental effects may have only persisted in traits submitted to weaker selection. Further, differences in the genetic basis of inbreeding depression could differ between the studied traits. If overdominance rather than dominance underlies the expression of inbreeding depression for fecundity or lice prevalence, the genetic load cannot be removed as heterozygote advantage always exerts a load at equilibrium (Dudash *et al*. 1997). Thus, both the inbreeding history of the study population and possible differences in the genetic basis of inbreeding depression between the studied traits could lead that malaria resistance, but not fecundity or prevalence of feather lice, to be uncoupled with genetic diversity in the study population (e.g. Dudash *et al*. 1997; Wiehn *et al*. 2002).

The genetic basis of the trait being considered is likely to affect the degree of its correlation with multilocus heterozygosity. Resistance to avian malaria parasites may be modulated by heterozygosity at some functional genes directly involved in immune defence rather than by heterozygosity across the whole genome (e.g. Westerdahl *et al*. 2005). If this was the case, we would expect a weak correlation between multilocus heterozygosity measured at neutral markers and the heterozygosity of the pool of genes controlling malaria resistance under identity disequilibrium because random effects due to Mendelian segregation do not depend on the number of typed marker loci only, but also on the number of loci effectively involved in the correlation (Aparicio *et al*. 2007). Although resistance to malaria parasites is known to be a highly polygenic trait (Hill 1998), we may have had low power to estimate heterozygosity at such specific genes using a small number of neutral markers (Balloux *et al*. 2004; Aparicio *et al*. 2007). Another possibility is that increased genome-wide heterozygosity shows advantages in multiparasite infections but has little or no effect on resistance to single-strain infections such as the one studied here (Taylor *et al*. 1998; McClelland *et al*. 2003; Wedekind *et al*. 2006). Finally, malaria resistance could be associated with certain alleles from functional genes rather than with heterozygosity per se and, thus, malaria-mediated heterozygote advantage could be simply low (e.g. Hill *et al*. 1991; Hill *et al*. 1992; Gaikwad *et al*. 2005; Thathy *et al*. 2005; Bonneaud *et al*. 2006; Wedekind *et al*. 2006).

The correlative nature of our study has some limitations that may have obscured any possible association between individual heterozygosity and prevalence of malaria parasites. The severest fitness consequences of avian malaria generally occur just after the first exposure to the parasite (i.e. the acute infection) whereas individuals surviving the primary infection can either clear it or carry chronic stages that generally have few, if any, effects on the infected host (Bensch *et al*. 2007; Kilpatrick *et al*. 2006). Given that severely ill individuals are not generally sampled and individuals dead from natural causes are almost impossible to detect in the wild, our data sets and those of other studies analysing malaria prevalence in natural populations mainly consist of individuals that have survived the acute infection and carry chronic infections and those birds without malaria parasites either because they have never been exposed to the parasite or because they have handled the infection and cleared it (Westerdahl *et al*. 2005). Accordingly, the infection intensities of the studied lineages are generally below the limit of detection of methods based on microscopy examinations of blood smears (Ortego *et al*. 2007b), suggesting levels of parasitemia that characterize chronic infections (Jarvi *et al*. 2003). Thus, interpreting data on malaria parasite prevalence may be challenging, and our finding of no association between microsatellite variation and prevalence of malaria parasites in the study host–parasite system may be a consequence of the weakness of adopting an observational approach (Côté *et al*. 2005; Westerdahl *et al*. 2005; Bensch *et al*. 2007). Experimental approaches could help to disentangle these questions by following the whole course of infections, determining whether individuals have either recovered or died from acute infections, and controlling for variability in prevalence that is merely due to spatial and temporal differences between hosts in exposure to infection (Hawley *et al*. 2005).

Up to now, we have assumed that malaria infections have detrimental fitness impact in lesser kestrel populations. However, another possibility is that both primary and chronic malaria infections had little or no impact on fitness in the study species and this could explain the absence of association between individual genetic diversity and blood parasite prevalence (e.g. Kilpatrick *et al*. 2006; Bensch *et al*. 2007). Furthermore, the very low parasite prevalence observed in lesser kestrels (less than 5% of infected birds; Ortego *et al*. 2007b) may have also reduced the effective power of our statistical analyses to detect a relationship between heterozygosity and infection status with so few individuals in the infected group, in spite of the number of birds screened was very high.

In conclusion, despite a large sample size and an extensive data set, we have found no correlation between individual heterozygosity and prevalence of avian malaria parasites. These results suggest that research on the genetic basis of resistance to malaria parasites should try to target candidate genes, their products or markers tightly linked to them, thus, pushing research in this area in the direction of genome mapping, transcription profiling, and proteomics as these methodologies become easier to apply to nonmodel organisms (e.g. Acevedo-Whitehouse & Cunningham 2006). Further studies should also consider estimates of the intensity of avian malaria infections which might help to identify the factors influencing parasite resistance (e.g. MacDougall-Shackleton *et al*. 2005), although such estimates can be only provided by ocular screening of blood smears, which can severely underestimate the prevalence of blood parasites, or using more complex and costly methods such as real-time PCR techniques. Undoubtedly, experimental studies are also necessary to get a better understanding on the correlation between heterozygosity and susceptibility to diseases (e.g. Hawley *et al*. 2005; Wedekind *et al*. 2005; 2006), although these approaches may be unfeasible in endangered species, such as the lesser kestrel, if they entail important risks and raise ethical issues difficult to address (Hale & Briskie 2007).

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