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MAJOR HISTOCOMPATIBILITY ALLELES ASSOCIATED WITH LOCAL RESISTANCE TO MALARIA IN A PASSERINE

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Abstract.—Malaria parasites are a major cause of human mortality in tropical countries and a potential threat for wildlife, as witnessed by the malaria-induced extinction of naive Hawaiian avifauna. Identifying resistance mechanisms is therefore crucial both for human health and wildlife conservation. Patterns of malaria resistance are known to be highly polygenic in both humans and mice, with marked contributions attributed to major histocompatibility (*Mhc*) genes. Here we show that specific *Mhc* variants are linked to both increased resistance and susceptibility to malaria infection in a wild passerine species, the house sparrow (*Passer domesticus*). In addition, links between host immunogenetics and resistance to malaria involved population-specific alleles, suggesting local adaptation in this host-parasite interaction. This is the first evidence for a population-specific genetic control of resistance to malaria in a wild species.

Key words.—Avian malaria, local adaptation, major histocompatibility complex, resistance alleles, susceptibility alleles, wild passerine.

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The end of the 20th century witnessed the emergence of highly infectious diseases in wildlife. Alarmingly, evidence now suggests that human-induced climate change affects the incidence and geographic range of arthropod-borne diseases, such as those carried by mosquitoes (Benning et al. 2002). As a result, we expect more susceptible hosts to be infected as temperatures increase (Daszak and Cunningham 2000). Malaria parasites are a major cause of human mortality in tropical regions (Snow et al. 2001) and have been involved in the loss of several species of endemic Hawaiian birds (Benning et al. 2002). It is therefore urgent to identify the mechanisms of malaria resistance in wildlife, with the hope of buffering the impacts anticipated from the expansion of malaria to new parts of the world.

The pathogenic effects of malaria parasites on avian hosts (reviewed in Atkinson 1999) have mainly been established from postmortem examinations of dead birds (Beier et al. 1981; McConkey et al. 1996) and from experimental infections in captivity (Atkinson et al. 2001; Garvin et al. 2003). Only a few studies have provided evidence for the fitness consequences of malaria infections on wild populations of birds (Richner et al. 1995; Oppliger et al. 1996; Dawson and Bortolotti 2000; Merino et al. 2000; Sol et al. 2003; but see Siikamäki et al. 1997). Some of the first examples came from studies on great tits (*Parus major*) and demonstrated a trade-off between reproduction and defense against infections with *Plasmodium* parasites (Richner et al. 1995; Oppliger et al. 1996). Another study showed that body condition and intensity of *Haemoproteus* infection were negatively correlated in both male and female American kestrels (*Falco sparverius*) during incubation, suggesting either the existence of host-parasite competition for access to host resources, increased host investment in immune defense, tissue repair, or higher susceptibility of individuals in poor condition (Dawson and Bortolotti 2000). Furthermore, female return rates declined with the intensity of *Haemoproteus* infection, although their disappearance could be attributed either to dispersal or to death. Sol et al. (2003) showed that malaria could result in death by demonstrating that juvenile feral pigeons (*Columba livia*) with high parasitemia of *Haemoproteus* protozoa had lower chances of survival to adulthood relative to nonparasitized individuals.

Many genes are thought to be involved in resistance to malaria in humans (reviewed in Hill 1998), with a marked contribution of the major histocompatibility (*Mhc*) genes. Indeed, both *Mhc* class I and *Mhc* class IIB alleles (HLA-B*5301 and HLA-DRB1*1302, respectively) seem to protect Gambian children from infections with severe malaria (Hill et al. 1991). Yet, so far few studies have examined variation in host genes in relation to susceptibility to infectious diseases in wild vertebrates. The major histocompatibility complex (*Mhc*) is particularly well suited for examining such associations, as it codes for highly polymorphic molecules responsible for eliciting specific immune responsiveness when bound to foreign peptides.

Diversity in this area of the genome is thought to be in part maintained by pathogen-driven selection, based on the coevolutionary arms race taking place between hosts and pathogens. As a result, we expect close associations between candidate *Mhc* alleles and altered disease susceptibilities.

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Previous studies have shown that specific *Mhc* alleles could be associated to resistance or susceptibility to infectious diseases in the wild (e.g., resistance: Paterson et al. 1998; Westerdahl et al. 2005; resistance and susceptibility: Schad et al. 2005), as predicted by processes such as negative frequency-dependent selection. But only a few studies have experimentally verified the link between naturally occurring pathogens and *Mhc* alleles in natural populations, and these are limited to studies of fish species (Hedrick et al. 2001; Langefors et al. 2001; Arkush et al. 2002; Wegner et al. 2003).

We chose to examine the prevalence of malaria infections in two wild populations of house sparrows (*Passer domesticus*) in relation to the diversity found at the most variable *Mhc* class I gene family (Bonneaud et al. 2004), and in association with the presence or absence of specific *Mhc* class I alleles. MHC class I molecules are ubiquitously expressed at the surface of all cells. They serve to present foreign peptides from intracellular pathogens (virus or intracellular protists) to circulating cytotoxic Th8+ lymphocytes and thereby initiate immune clearance (Janeway et al. 1999). Malaria parasites are found in hosts as gametocytes or sporozoites, which invade erythrocytes in the blood stream and other cells in different host tissues, so peptides produced by the degradation of malaria parasites are likely to be presented by MHC class I molecules. By comparing two wild populations of house sparrows that are geographically separated by several hundred kilometres, we were able to examine the effects of differing pathogenic pressures on host genes and how the environment can act to modulate host-parasite coevolutionary processes.

In addition to assessing *Mhc* genotypes, we screened individuals at neutral microsatellite loci to determine the genetic distance existing between our two populations and to serve as a control for divergence between populations at *Mhc* loci likely to be under selection. In a previous study, we found that *Mhc* sequences displayed a ratio of nonsynonymous to synonymous substitutions greater than one, supporting the idea that *Mhc* alleles are under long-term selection in the house sparrow (Bonneaud et al. 2004). In contrast to *Mhc* loci, microsatellites are assumed to behave neutrally, and variation at these markers is thought to be primarily driven by nonselective evolutionary factors such as genetic drift, gene flow, and mutation (Nei 1987). Therefore, we can also estimate selection at *Mhc* alleles by comparing the variation found at both neutral and *Mhc* loci.

Materials and Methods

Study populations

We studied 208 house sparrows from two populations in France (Chizé, $n = 144$; Foljuif, $n = 64$) located about 400 km apart. The first population consists of wild individuals belonging to a nest-box population established in 1992 at the Centre d'Etude Biologique de Chizé. A large proportion of the birds are color banded. The total area of the site of Chizé is relatively small (3 ha), so that all individuals feed, roost, and nest in very close proximity to one another. The second population consists of 63 wild house sparrows (31 females and 32 males) temporarily housed in outdoor aviaries ($3 \times 2 \times 2.5$ m) at the Station Biologique de Foljuif. Birds were maintained in groups of four to six individuals with ad libitum access to food (a commercial mixture of seeds) and water. Blood samples were collected for each individual and stored at -20°C until analysis in the laboratory.

Screening for malaria infections

We used a highly efficient nested polymerase chain reaction (PCR; Waldenström et al. 2004) to amplify 479 bp of the cytochrome *b* of both *Plasmodium* and *Haemoproteus* parasites from infected birds. Negative infections were confirmed by repeated PCR and amplification of *Mhc* loci was used as a control for sample quality. We identified strains by sequencing the fragments (BigDye [R] ver. 1.1 sequencing kit, Applied Biosystems, Foster City, CA) on an ABI PRISM 3100 sequencing robot. Unresolved sequences showing double peaks in the electropherograms were examined for putative multiple infections by cloning (TOPO-cloning kit, Invitrogen, Carlsbad, CA) and sequencing (Pérez-Tris and Bensch 2005a).

Mhc screening

We screened all individuals to assess allelic diversity at the most variable *Mhc* class I gene family using the PCR-based DGGE method. This method allows us to examine single-nucleotide polymorphism at *Mhc* class I exon 3, corresponding to the highly variable peptide binding site of the protein (α2 domain). The PCR primers used were GCA21M-fA23M. Each DGGE band is considered to correspond to one allele (Bonneaud et al. 2004).

This genotyping method did not allow us to determine the level of heterozygosity present at each individual locus. Instead, it gave us an estimate of the overall number of alleles present in the most variable lineage of *Mhc* class I genes. Individuals who carry the largest numbers of *Mhc* alleles were considered the most diverse. Although there seems to be a maximal number of six *Mhc* loci (the most diverse individuals had 11 alleles), we could not rule out the possibility that we were amplifying a gene family encompassing more than six loci.

Microsatellite analyses

Individuals were genotyped using seven microsatellite markers: Pdo3, Pdo4, Pdo5, Pdo6 (Griffith et al. 1999), Mjg1 (Shou-Hsien et al. 1997), Fhu2 (Primmer et al. 1996), and Ase18 (Richardson et al. 2000). Amplifications were run in a final volume of 10 μl including 15–50 ng of DNA, 50–200 nM of each primer, 300 μM of dNTPs, 1 μl of 10X incubation buffer (50 mM KCl, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 0.1% TritonX-100, pH 9.0) and 0.25U of *Taq* DNA polymerase (Qbiogene, Irvine, CA). The reaction was performed in a Gene Amp PCR System 9700 thermocycler (Applied Biosystems). Samples were then run in an ABI 310 automated sequencer. Allele sizes were determined using GENESCAN software version 2.1 (Applied Biosystions) with reference to the GENESCAN ROX 500 size standard.

Population structure and measures of relatedness

We calculated the average number of alleles (n_a), the observed and expected heterozygosity (H_O and H_E), and the

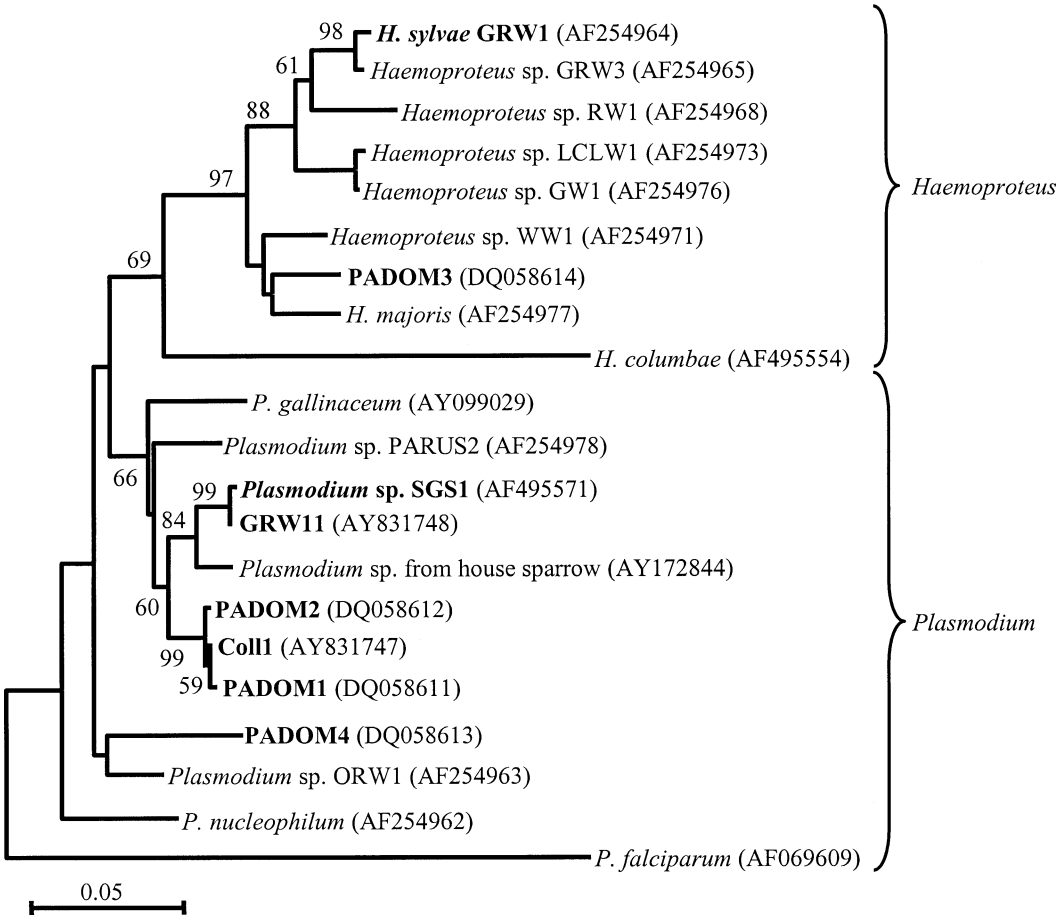

FIG. 1. Phylogenetic position of the eight malaria parasites found in the house sparrow (*Passer domesticus*) populations of Chizé and Foljuif (in bold), based on cytochrome *b* sequences. The tree has been obtained using the neighbor-joining method and a Jukes-Cantor model of nucleotide substitution, with $\alpha = 0.45$ as estimated from the data. Sequences from known avian parasites were included for comparison, and human *Plasmodium falciparum* was used as an outgroup. GenBank accession numbers of all sequences are indicated. Numbers on interior branches indicate bootstrap support (1000 replications, only values over 50% are shown).

inbreeding coefficient (F_{IS}) for each locus and for both populations. We used Guo and Thompson's (1992) method to detect significant departure from Hardy-Weinberg equilibrium (Fisher's exact test with the Markov chain method, chain length = 1000 and dememorizations steps = 1000). We determined the pairwise linkage disequilibrium between loci for each population to assess independence. We calculated the genetic differentiation between populations using an exact test, and the joint probability over all loci was obtained using Fisher's combined probability tests (Sokal and Rolf 1995). All these analyses were performed using Genepop version 2.0 (Raymond and Rousset 1995). The pairwise F_{ST} statistic between the two populations was calculated using Arlequin version 2.00 (Schneider et al. 2000).

Statistical analyses

The association between specific *Mhc* alleles and the infection status was assessed using a multivariate generalized linear model, which included the presence/absence of the seven most common alleles. The inclusion of the most common alleles in the same model allowed us to avoid multiple pairwise tests. Statistical analyses were done using SAS statistical software (SAS Institute 1999).

RESULTS

Considering all lineages together, the prevalence of malaria infection did not differ between the two house sparrow populations (generalized linear model: $\chi^2 = 2.66$, $P = 0.103$, $N_{\text{Chizé}} = 68\%$, $N_{\text{Foljuif}} = 56\%$). Eight mitochondrial malaria lineages were found (six *Plasmodium* and two *Haemoproteus* strains; Fig. 1) with prevalence ranging from 54% to 0.5% (Fig. 2). The prevalence of the most common parasite strain (*Plasmodium* sp., strain SGS1) differed significantly between the two populations (Chizé: 86/144, Foljuif: 26/64, $\chi^2 = 6.5$, $P = 0.011$). The other strains were too rare to test population differences (prevalence $< 9\%$).

Microsatellite Analyses

We report the results for the genetic variation of both populations in Table 1. Two loci, Fhu2 in Chizé and Pdo4 in Foljuif, were found to be significantly deficient in hetero-

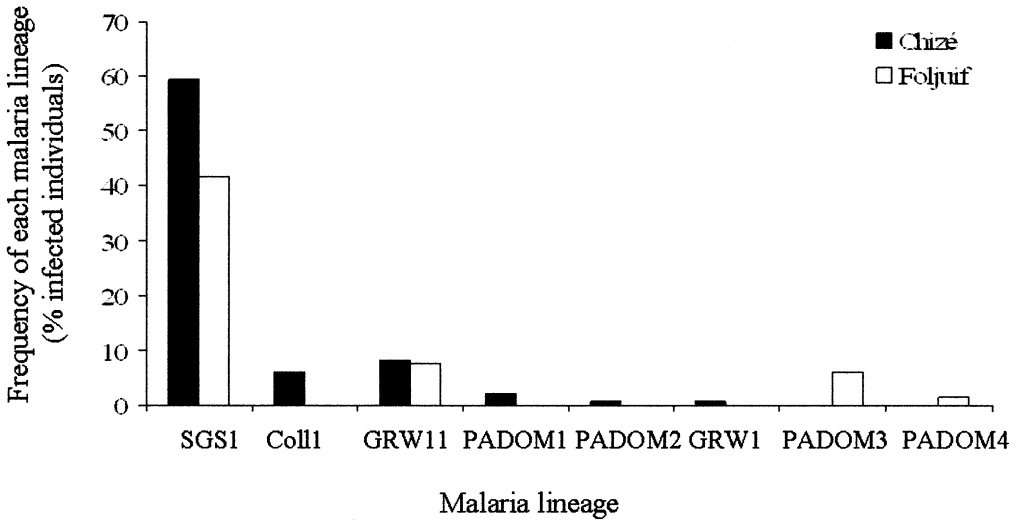

FIG. 2. Frequency of each malaria lineage in percent individuals infected in each population (Chizé and Foljuif).

zygotes after sequential Bonferroni correction (Rice 1989). However, neither locus was deficient in both populations, suggesting that the deficiency is not the result of null alleles. Only three of 42 tests of linkage disequilibrium were found to be significant after sequential Bonferroni correction, and all involved the Fhu2 locus. For this reason, we decided to remove Fhu2 from all further analyses. The two populations were found to be significantly differentiated (exact test, $P < 0.0001$) and displayed a very small but significant F_{ST} ($F_{ST} = 0.004$, $P < 0.01$). The significance of this F_{ST} value arises from the fact that both populations have a very large number of microsatellite alleles (>85) at two of the six loci used in the analysis (57% alleles at the Pdo4 and Pdo6 loci were found in less than 10% of individuals), many of which are rare or absent in one of the two populations. Thus, while the F_{ST} is significant, differentiation of the two populations is extremely weak. This is supported by the fact that if we remove Pdo4 and Pdo6, the F_{ST} value becomes nonsignificant ($F_{ST} = 0.002$, $P = 0.099$).

Mhc Genotypes and Malaria Infections

We found 47 different *Mhc* class I alleles in all. Because most alleles were rare (40 of 47 were found in less than 10% of individuals), we focused on the seven most common. The mean number of alleles per individual did not differ between populations (Chizé: mean ± SE = 3.34 ± 2.02, Foljuif: 3.23 ± 1.77; $F_{1,206} = 0.13$, $P = 0.718$).

Three of the seven most common alleles had population-specific effects either in terms of increased resistance or susceptibility to the SGS1 strain (multivariate generalized linear model: population × *a151*, $\chi^2 = 5.62$, $P = 0.018$; population × *a161*: $\chi^2 = 5.53$, $P = 0.019$; population × *a172*: $\chi^2 = 4.11$, $P = 0.043$; Fig. 3). Alleles *a151* and *a172* were as-

TABLE 1. Neutral variation at the seven microsatellite loci in both populations. n_a, number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; F_{IS}, inbreeding coefficient.

Population	n_a ± SD	H_O ± SD	H_E ± SD	F_{IS}
Chizé				
Pdo3	17	0.888	0.886	−0.003
Mjg1	24	0.963	0.933	−0.032
Pdo4	99	0.925	0.987	+0.063
Fhu2	15	0.664	0.816	+0.188
Pdo5	16	0.916	0.886	−0.034
Ase18	18	0.822	0.831	+0.010
Pdo6	85	0.953	0.981	+0.028
All loci	39.14 ± 33.75	0.876 ± 0.105	0.903 ± 0.067	0.031
Foljuif				
Pdo3	18	0.803	0.882	+0.090
Mjg1	21	0.918	0.931	+0.015
Pdo4	98	0.918	0.984	+0.068
Fhu2	14	0.672	0.768	+0.126
Pdo5	15	0.836	0.862	+0.030
Ase18	18	0.754	0.873	+0.137
Pdo6	90	0.951	0.981	+0.031
All loci	39.14 ± 34.82	0.836 ± 0.101	0.897 ± 0.076	0.071

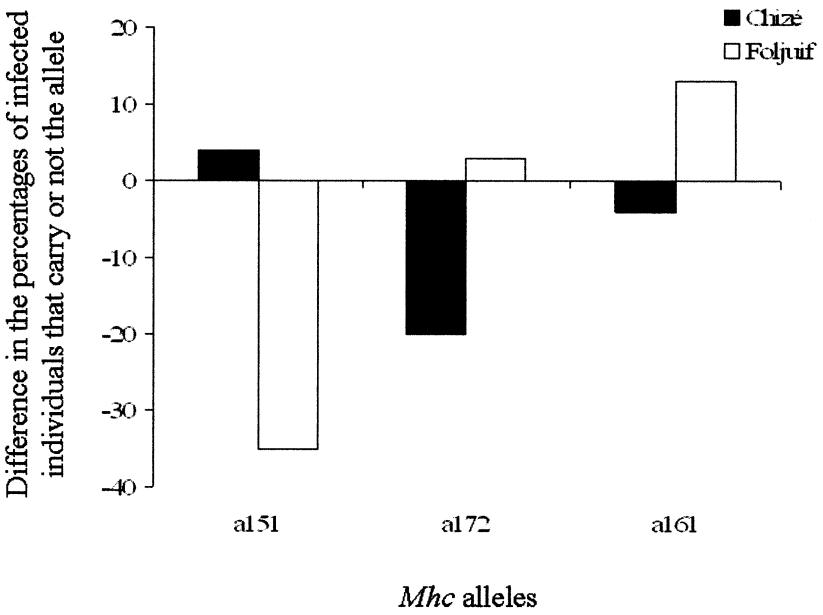

FIG. 3. Difference between the percentage of infected individuals carrying the *Mhc* allele and the percentage of infected individuals not carrying the allele, for the two populations of house sparrows (*Passer domesticus*). Positive values represent increased susceptibility to infection linked to the presence of the *Mhc* allele, whereas negative values indicate increased resistance associated to the allele.

sociated with higher resistance in Foljuif and Chizé, respectively, whereas *a161* was linked to increased susceptibility in Foljuif only.

Multiple strain infections, which always involved SGS1 plus another parasite lineage, occurred in 11.7% (14/120) of all infected individuals. Their occurrence was also associated to one *Mhc* allele because none of the individuals with the *a163* allele carried infections with two malaria strains ($\chi^2 = 6.57$, $P = 0.010$). Remarkably, multiple infections never occurred in Foljuif (Fisher's exact test, $P = 0.021$), where the *a163* allele was twice as frequent as in Chizé (44% vs. 19%). This was the only common *Mhc* allele whose frequency differed between the two sites ($\chi^2 = 13.3$, $P = 0.0003$).

DISCUSSION

Today, parasites responsible for avian malaria are a major threat to endangered populations persisting in remote areas. For conservation purposes, it is urgent to find out whether there are immunogenetic variants that may influence susceptibility to these pathogens. Moreover, there are still very few studies examining the genetic basis of resistance to naturally occurring pathogens in wild vertebrate populations. We show here that *Mhc* class I alleles may be associated with increased resistance to malaria in wild bird populations. By comparing two populations of wild house sparrows, we identified two different alleles associated with protection against the same malaria strain, suggesting local adaptation in this host-parasite system.

Avian malaria parasites are known to potentially exert strong selection pressures on their hosts, as revealed by experimental infections (Atkinson et al. 1995), treatment with antimalaria drugs (Merino et al. 2000), and the damages resulting from human-induced spread of malaria to the Hawaiian archipelago (Benning et al. 2002). However, despite the potential ecological and evolutionary role of malaria parasites in shaping host life histories, we still lack knowledge of the host genes implicated in resistance. Studies on humans and laboratory mice have stressed the importance of *Mhc* as well as non-*Mhc* genes as determinants of either resistance or susceptibility to *Plasmodium* (Jayawardena et al. 1983; Hill et al. 1991; Hill 1998; Fortin et al. 2002), or of the chronic sustainability of mixed malaria infections (de Roode et al. 2004). Here we show that *Mhc* class I alleles are associated with the incidence of malaria infections in a wild species.

We found that alleles associated either with reduced or increased risk of infection were population specific. This result is reminiscent of studies examining the HLA-based resistance to malaria in humans (Hill 1998). One *Mhc* class I (HLA-BRB1*5301) and one *Mhc* class IIB (HLA-B*1302) allele were found to be associated with resistance to severe malaria in Gambian children (Hill et al. 1991). However, a study of severe malaria in Kenya revealed that the *Mhc* class I allele HLA-BRB1*0101, and not the other two previously identified in The Gambia, was associated with protection against the disease (Yates 1995). Such an apparent discrepancy may be explained by the occurrence of separate genetic recombination events in the parasite lineage between the two sites, resulting in the selection of different host genes specifically directed against local pathogenic epitopes (Hill et al. 1998).

Experimental cross-infections between the two populations of house sparrows should help us elucidate potential local adaptation processes in this host-parasite system. At present, there is circumstantial evidence that the *Plasmodium* lineage SGS1 has an extraordinary potential for local adaptation. This parasite can colonize different house sparrow populations by host switching from other bird species (SGS1 has been ob-

served in 19 passerine species sympatric to house sparrows; J. Pérez-Tris, unpubl. data) that can transport the parasites between distant areas connected by migratory routes (Pérez-Tris and Bensch 2005b). If the establishment of an infection depends on the match between host *Mhc* genes and parasite genes linked to infectivity, parasite dispersal may enhance local parasite adaptation, because immigrant parasite alleles will help track local host *Mhc* alleles (Dybdahl and Storfer 2003). A recent study of blackcaps (*Sylvia atricapilla*) supported such a scenario by showing that SGS1 displays both a superior ability to disperse among host populations and a high local infection success (Pérez-Tris and Bensch 2005b).

Analyses of variation at neutral genetic markers showed that the two populations were very weakly differentiated ($F_{ST} = 0.004$). However, the significance of the F_{ST} value obviously results from the stochastic distribution between both populations of the rare alleles found at the two most highly polymorphic loci. Differences in the associations of *Mhc* alleles to resistance/susceptibility to malaria between the two populations are therefore unlikely the result of nonselective evolutionary forces such as drift. This finding corroborates previous work that showed a higher rate of nonsynonymous to synonymous substitutions in *Mhc* sequences, supporting the idea that *Mhc* alleles are under long-term selection in the house sparrow (Bonneaud et al. 2004).

The spread of pathogens due to global climate changes and human-induced opening of wild areas are often evoked as a potential threat for wildlife (Daszak and Cunningham 2000). To evaluate such a threat, it is important to know the susceptibility of populations to new diseases, as well as their potential to evolve resistance mechanisms (Woodworth et al. 2005). Thus, better understanding of the genetic background of pathogen resistance in natural populations is likely to be crucial in wildlife management.

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