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LETTER

Antagonistic effects of a *Mhc* class I allele on malaria-infected house sparrows

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Abstract

Genes of the Major Histocompatibility Complex (*Mhc*) play a fundamental role during the immune response because MHC molecules expressed on cell surface allow the recognition and presentation of antigenic peptides to T-lymphocytes. Although *Mhc* alleles have been found to correlate with pathogen resistance in several host-parasite systems, several studies have also reported associations between *Mhc* alleles and an accrued infection risk or an accelerated disease progression. The existence of these susceptibility alleles is puzzling, as the cost generated by the infection should rapidly eliminate them from the population. Here, we show that susceptibility alleles may be maintained in a population of house sparrows (*Passer domesticus*) if they have antagonistic effects on different malaria parasites. We found that one *Mhc* class I allele was associated with a 2.5-fold increase in the risk to be infected with a *Plasmodium* strain, but with a 6.4-fold reduction in the risk to harbour a *Haemoproteus* strain. We suggest that this antagonistic effect might arise because *Mhc* genes can alter the competitive interactions between malaria parasites within the host.

Keywords

Antagonistic pleiotropy, avian malaria, co-evolution, house sparrow, parasite competition, *Passer domesticus,* susceptibility, resistance.

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INTRODUCTION

Understanding how variation of selected genes is maintained has been a major challenge for evolutionary biologists (Hedrick 1972; Maruyama & Nei 1981; Hedrick & Thompson 1983; Hughes & Nei 1992). *Mhc* genes are a particularly intriguing case as this complex of genes is one of the most polymorphic regions of the vertebrate genome and plays an important role in parasite resistance (Segal & Hill 2003; Frodsham & Hill 2004; Nikolich-Žugich *et al.* 2004). MHC molecules present peptides derived from pathogens to cytotoxic T-cells (CTLs). If the complex MHC-peptide binds to a T-cell receptor (TCR), CTLs are activated and MHC-restricted CTL clones expand to control the infection (Parham & Ohta 1996; McDevitt 2000). Polymorphic class I and II loci ensure the presentation of a broad spectrum of antigenic peptides and several examples of associations between *Mhc* alleles and resistance to infectious diseases have been reported in humans (Hill *et al.* 1991; Meyer *et al.* 1994; Siddiqui *et al.* 2001; Fitness *et al.* 2002), animal models (Briles *et al.* 1977; Penn *et al.* 2002; McClelland *et al.* 2003), and wildlife species (Paterson *et al.* 1998; Lohm *et al.* 2002; Wegner *et al.* 2003; Westerdahl *et al.* 2005; Bonneaud *et al.* 2006). Surprisingly, however, *Mhc* alleles are almost equally often reported to be associated with increased susceptibility or increased resistance to infection (Segal & Hill 2003; Frodsham & Hill 2004; Schad *et al.* 2005; Bonneaud *et al.*

2006). For instance, several alleles have been associated with accelerated or retarded progression of HIV-1 infection to AIDS (Carrington *et al.* 1999; Hendel *et al.* 1999). The persistence of susceptibility alleles poses an evolutionary dilemma as the costs associated with carrying the alleles should eliminate them from the population (O'Brien & Carrington 1999; Wills 1999). In theory, alleles that have antagonistic effects on fitness can be maintained in the population (Zhang *et al.* 2002). Classical examples of such pleiotropic genes involve haemoglobin and enzymatic disorders, such as the G6PD deficiency (Tishkoff & Verrelli 2004). Here, we report a case of a *Mhc* class I allele that is associated with increased risk of harbouring an infection with a *Plasmodium* strain and an enhanced chance of being free of a *Haemoproteus* parasite in the same population of house sparrows (*Passer domesticus*).

MATERIALS AND METHODS

Screening for malaria infections

A population of house sparrows, a sedentary passerine bird, located in North-East France (Crégy lès Meaux; 48°58′N, 2°52′E) was followed from 2004. Eighty five individuals were captured with mist nets and blood samples were collected by vein puncture (24 samples in 2004, 40 in 2005 and 21 in 2006). We used a highly efficient nested polymerase chain reaction (PCR) to amplify a 524-bp-long fragment of the cytochrome *b* of both *Plasmodium* sp. and *Haemoproteus* sp. parasites from infected birds (Waldenström *et al.* 2004). This method is highly repeatable, and has a detection limit identifying parasitemia as low as one infected red blood cell per 100 000. We identified parasite strains by sequencing the fragments on an ABI3730XL, Applied Biosystems, Cartaboeuf, France. Sequence electropherograms were carefully inspected for double nucleotide peaks. Although very effective, this screening method has been suggested to underestimate the occurrence of multiple infections (Valkiûnas *et al.* 2006). If one parasite strain has a much higher intensity compared with other strains co-infecting a given host, then there is a risk that the nested PCR will only amplify the fragment belonging to the most abundant strain (Pérez-Tris & Bensch 2005). To avoid this potential problem we also used strain specific primers for the *Plasmodium* SGS1 strain (SGS1F: 5′-ATGTGGTGGATATCTTGTTGTAAGTGAC; SGS1R: 5′-ATATTTTAAGTCTTGATATTAAAGGA) and the *Haemoproteus* PADOM3 strain (PADOM3F: 5′-TTGTGGAGGATATACTATTAGTGAT; PADOM3R: 5′-GTGTATTATGTCTAGATATAAAAGGA). We used the same pre-amplification (20 cycles) using primers located outside the target fragment (HaemFN and HaemRN2) followed by a final amplification (35 cycles) with the strain specific primers.

Screening of *Mhc* class I variation

We screened all individuals to assess allelic diversity (number of alleles per individual ranging from one to eight) at the most variable *Mhc* class I gene family using the PCR-based denaturant gradient gel electrophoresis (DGGE) method (Myers *et al.* 1987). 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), but does not allow us to assign the alleles to specific loci (Bonneaud *et al.* 2004). To preferentially amplify transcribed alleles, we used cDNA sequences as reference [GCA21M 5′-CGTACAGCGGCTTGTTGGCTGTGA-3′ and fA23M 5′-GCGCTCCAGCTCCTTCTGCCCATA-3′ (Bonneaud *et al.* 2004)]. The polymerase chain reaction products were separated using a DGGE. The DGGE gel contained 7% of 40% 19:1 acrylamide/bisacrylamide, 1x TAE, formamide, and a 40–65% denaturating gradient of urea. Standardized markers were used to enable comparison between gels. The gels were run at 60 °C in 1x TAE buffer for 20 h at 180 V. As mentioned by Westerdahl *et al.* (2004), the DGGE method does not have a 100% resolution and therefore we could have failed to separate some alleles. However, as suggested by two recent studies (Richardson & Westerdahl 2003; Westerdahl *et al.* 2004), this screening method is more repeatable and sensitive in detecting genetic variation than the RFLP method.

DGGE bands were excised from gels and re-amplified with the same primers to be sequenced. Then, we translated the nucleotide sequence into the corresponding amino acid sequence with Expasy Translate Tool and checked if the sequence presented conserved domains in GenBank. The allele *pado123* (which was associated with malaria prevalence) had the following sequence (GenBank accession no. EF429132): 5′CGGTTGTTTGGCTGTGACCTCCTATCCAATGGGAGCGTCCGTGGATCCCGCCGGGAGGGCTACGATGGGCAGGATTTCATCTCCTTTGACCTGGAATCCGGGAGATTTGTGGCGGCCGACAACGTTGCTGAGATCACCAGGAGGCGCTGGGAACAGGAAGGGACTGTGGCTGAGAGGTGGACGAATTACCTGACGGTTGTTTGGCTGTGACCTCCTATCCAATGGGAGCGTCCGTGGATCCCGCCGGGAGGGCT 3′.

The corresponding amino acid sequence (GCDLLSNGSVRGSRREGYDGQDFISFDLESGRFVAADNVAEITRRRWEQEGTVAERWTNYLKHECPEWLRKYVGYGQKELER) fits with conserved domains of the MHC class I protein complex (GenBank, pfam0019, MHC, class I Histocompatibility antigen domains alpha1-2).

Microsatellite analyses

Thirty seven individuals (16 *pado123*$^{+}$ and 21 *pado123*$^{-}$) were genotyped using seven microsatellite markers: Pdo3, Pdo4, Pdo5, Pdo6 (Griffith *et al.* 1999), Mjg1 (Li *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% Triton X-100, pH 9.0) and 0.25 U of *Taq* DNA polymerase (Qbiogene, Irvine, CA, USA). The reaction was performed in a Gene Amp PCR System 9700 thermocycler (Applied Biosystems). Fluorescently labelled fragments were analysed in an ABI 310 automated sequencer following the manufacturer's protocol using GENEMAPPER 3.0 software. Individual pairwise coefficients of relatedness were based on microsatellite genotype similarity. Using SPAGeDi 1.2 (Hardy & Vekemans 2002), three different relatedness coefficients were computed according to Queller & Goodnight (1989), Li *et al.* (1993) and Wang (2004).

Statistical analyses

The association between specific *Mhc* alleles and the infection status was tested in a multivariate GEE model [Proc GENMOD with binomial distribution of errors and logit link function, SAS Institute (1999)], which allowed us to test the interaction between the presence of the alleles and the parasite strain. The model included the eight most common alleles ($f > 15\%$), the total number of alleles, the season when birds were captured (winter or summer), the year, sex, and body mass. Because the total number of alleles can be nonlinearly related with the risk of parasitism (Wegner *et al.* 2003), we also included a squared term in the statistical model.

RESULTS

Based on cytochrome – *b* sequencing, we found three malaria lineages which ranged in prevalence from 31.8% (*Plasmodium* SGS1), to 14.1% (*Haemoproteus* PADOM3) and 3.5% (*Plasmodium* GRW11). Because *Plasmodium* GRW11 occurred in only three individuals we restricted the subsequent statistical analyses to *Plasmodium* SGS1 (hereafter *Plasmodium*) and *Haemoproteus* PADOM3 (hereafter *Haemoproteus*) infected birds. None of the 85 hosts was co-infected (harboured both *Plasmodium* and *Haemoproteus* strains) based on the nested PCR. However, when using the strain specific primers, it turned out that 6 out of 85 sparrows (7.1%) were co-infected by the *Plasmodium* and the *Haemoproteus* strains. This is not statistically different from the random expectation on the proportion of co-infected birds (6.2%, $P > 0.05$). Therefore, among the 85 individuals screened for malaria infections, 51% (43/85) were free of parasites, 28% (24/85) were only infected with the *Plasmodium* strain, 11% (9/85) were only infected with the *Haemoproteus* strain, and 7% (6/85) were co-infected. This finding confirms previous results showing that the nested PCR does actually underestimate the number of co-infected individuals, possibly amplifying only the most abundant parasite strain. Among the six co-infected birds, the nested PCR picked up the *Plasmodium* strain for three of them and the *Haemoproteus* strain for the other three. Instead of ignoring this information on the relative abundance of the two parasite strains in co-infected birds, we decided to analyse the data using two different approaches: first, we took advantage of the information on the relative abundance of the two strains (e.g. 27 individuals where the only or dominant parasite strain was *Plasmodium*; 12 individuals where the only or dominant parasite strain was *Haemoproteus*); second, we considered the co-infected birds as a separate group of individuals, which gave us four categories of birds (i.e. non-infected, infected with *Plasmodium*, infected with *Haemoproteus*, co-infected).

One allele (*pado123*, $f = 36.5\%$) was highly significantly associated with malaria prevalence, but the sign of the association depended on the parasite strain (parasite strain × *pado123*, $\chi^2_1 = 10.24$, $P = 0.0014$; Fig. 1). Because overdispersed data can give erroneously low *P*-values, we checked whether there was any indication of over- (or under-) dispersion in the data. The ratio deviance/d.f. was close to one (0.9497, $\chi^2_1 = 0.44$), showing that the data were not overdispersed.

The presence of *pado123* was associated with a decreased risk of being infected with *Haemoproteus* ($\chi^2_1 = 5.12$,

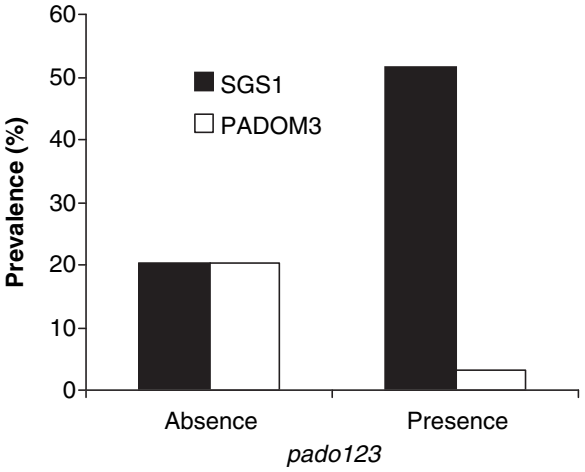

Figure 1 Prevalence of two lineages of malaria parasites (*Plasmodium* SGS1 and *Haemoproteus* PADOM3) as a function of the absence/presence of the allele *pado123*.

$P = 0.0236$; Fig. 1), but with an enhanced risk of carrying *Plasmodium* ($\chi^2_1 = 8.77$, $P = 0.0031$; Fig. 1). Prevalence did not differ between gender (*Plasmodium*, females: 31.0%; males: 33.3%, $\chi^2_1 = 0.045$, $P = 0.831$; *Haemoproteus*, females: 17.2%; males: 9.3%, $\chi^2_1 = 1.134$, $P = 0.286$). Similarly, body mass was not statistically associated with prevalence for any of the two parasites (*Plasmodium*, $\chi^2_1 = 0.080$, $P = 0.782$; *Haemoproteus*, $\chi^2_1 = 1.980$, $P = 0.159$). The total number of *Mhc* alleles was not a good predictor of the likelihood of harbouring the infection for any of the two parasite strains (*Plasmodium*: number of alleles $\chi^2_1 = 0.00$, $P = 0.9657$, squared number of alleles $\chi^2_1 = 0.13$, $P = 0.7203$; *Haemoproteus*: number of alleles $\chi^2_1 = 3.13$, $P = 0.077$, squared number of alleles $\chi^2_1 = 2.62$, $P = 0.1055$). As well, the interaction between the number of alleles and parasite strain was not statistically significant (parasite strain × number of alleles, $\chi^2_1 = 1.99$, $P = 0.1581$; parasite strain × squared number of alleles, $\chi^2_1 = 3.58$, $P = 0.0585$). Finally, including the year in the statistical model did not change the results as the interaction parasite strain × *pado123* was still highly significant ($\chi^2_1 = 10.32$, $P = 0.0013$), whereas the year effect approached the significance threshold ($\chi^2_2 = 5.92$, $P = 0.052$). To corroborate the finding that the opposite effect of *pado123* on the prevalence of the two parasite strains was not due to between-year variability in allele frequency, we also checked whether the proportion of birds carrying the allele differed across years. We did not find any evidence for an inter-annual variation in *pado123* frequency ($\chi^2_2 = 1.58$, $P = 0.4549$). However, we should keep in mind that birds were only sampled during a 3-year period which might be insufficient to detect temporal variation in allele frequency (Westerdahl *et al.* 2004; Bensch *et al.* 2007). Analysing co-infected birds as a fourth category, provided very similar results as the interaction parasite strain × *pado123* was still statistically significant ($\chi^2_1 = 5.00$, $P = 0.0253$).

There was very large within year (seasonal) variation in *Haemoproteus* prevalence. Only two of 50 sparrows sampled in fall-winter were infected with *Haemoproteus* (4.0%) whereas the prevalence rose to 28.6% in spring-summer ($\chi^2_1 = 10.532$, $P = 0.0012$). The prevalence of *Plasmodium* was constant throughout the year (fall-winter: 32.0%; spring-summer: 31.4%; $\chi^2_1 = 0.003$, $P = 0.955$). Because of this strong seasonal effect on *Haemoproteus* prevalence, we reran the model on the association between *pado123* and prevalence separately for birds caught in the two seasons. Again, *pado123* was significantly associated with prevalence for birds caught in spring-summer with the sign of the association being significantly different between parasite strains (parasite strain × *pado123*, $\chi^2_1 = 4.82$, $P = 0.0281$; Fig. 2a). The low prevalence of *Haemoproteus* prevented us to run the same model for birds captured during fall-winter (Fig. 2b). The frequency of *pado123* did not differ between

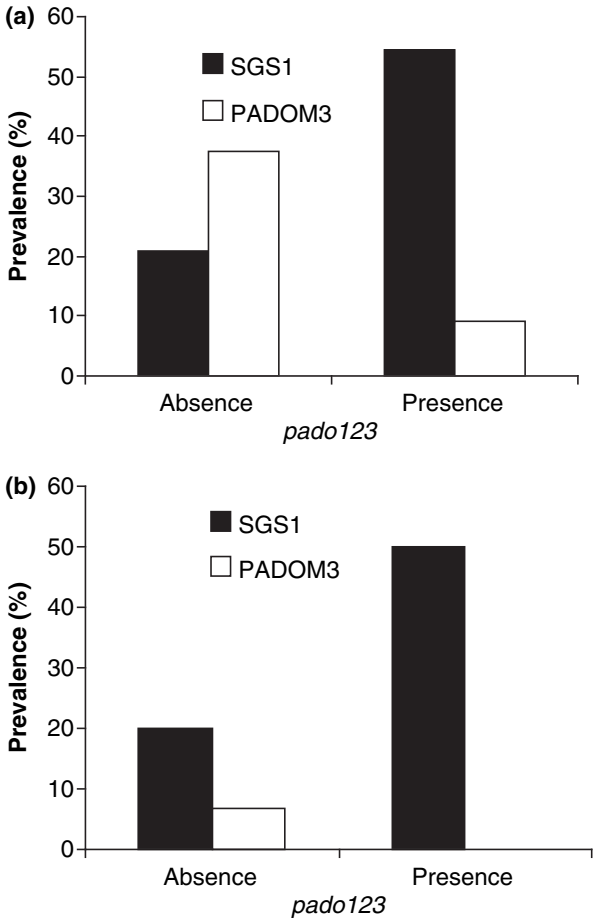

Figure 2 Prevalence of two lineages of malaria parasites (*Plasmodium* SGS1 and *Haemoproteus* PADOM3) as a function of the absence/presence of the allele *pado123* during the spring-summer (a) and fall-winter (b).

individuals captured in spring-summer (31.4%) and fall-winter (40.0%; $\chi^2_1 = 0.653$, $P = 0.419$). As for the overall sample, analysing co-infected birds as a separate group gave similar results (parasite strain × *pado123*, $\chi^2_1 = 4.00$, $P = 0.0455$).

Mhc alleles might co-occur in the same individual more often than expected by chance. We tested whether pairs of DGGE bands found in the sampled sparrows were in linkage disequilibrium (tended to co-occur more often than expected) but found no evidence for it (all $P > 0.05$). Similarly, positive/negative associations between *Mhc* alleles and malaria prevalence might arise because carriers of the allele have a common ancestor (are related to each other) and therefore share other genes that are casually involved in the observed associations. In this case, we would expect a greater pair-wise genetic relatedness between individuals sharing this *Mhc* allele. We performed a Mantel test to assess the association between a pair-wise relatedness matrix and a simple binary matrix (containing 0 for pairs

of individuals who shared or did not have the allele and one for pairs of individuals for which only one had the allele). There was no significant association between pairwise relatedness and binary matrices for the three relatedness estimators computed (all $P > 0.05$). This shows that the observed correlation between prevalence and the presence of the allele *pado123* is unlikely to have arisen as the consequence of other alleles shared by genetically related birds.

We also explored if and under which conditions the antagonistic effect of *pado123* could explain the maintenance of susceptibility. To do this we computed the fitness (W) as follows:

$$W = (1 - p_{\mathrm{Plasm}}) + [p_{\mathrm{Plasm}}(1 - c_{\mathrm{Plasm}})] \times (1 - p_{\mathrm{Haem}}) + [p_{\mathrm{Haem}}(1 - c_{\mathrm{Haem}})]$$

where p represents the proportion of birds infected and c the cost of the infection. W was computed independently for *pado123*$^+$ and *pado123*$^-$ birds, using the observed p_{Plasm} and p_{Haem} for each group of birds. We also computed W using prevalence values that were increased or decreased by a factor 2. The cost of the infection c was varied between 0.1 and 0.2 for *Plasmodium* and between 0.2 and 0.4 for *Haemoproteus*. We then plotted the relative fitness of *pado123*$^+$ birds ($W_{pado123^+}/W_{pado123^-}$) against the relative cost of being infected with *Plasmodium* or *Haemoproteus* ($c_{\mathrm{Plasm}}/c_{\mathrm{Haem}}$). For the observed prevalence values, *pado123*$^+$ birds achieve similar fitness than *pado123*$^-$ sparrows only when the cost of *Haemoproteus* infection is twice as high as the cost of *Plasmodium* infection. When the cost of *Haemoproteus* infection is similar to that of *Plasmodium*, the cost of susceptibility largely outweighs the benefits of resistance (Fig. 3).

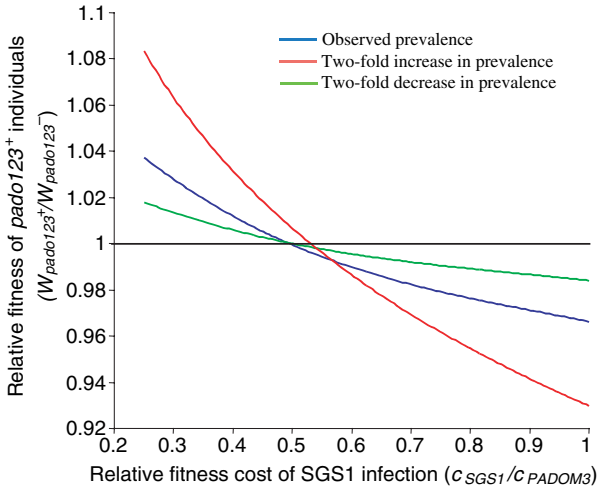

Figure 3 Relative fitness of birds carrying the *pado123* allele (W_{123^+}/W_{123^-}) as a function of the cost-inflicted by SGS1 and PADOM3 infections. *pado123*$^+$ birds achieve similar fitness than *pado123*$^-$ individuals only when the cost of PADOM3 infection is twice the one of SGS1 infection. This result holds for variable prevalence (blue line = observed prevalence; red line = increase in prevalence in a factor of 2; green line = decrease in prevalence in a factor of 2).

DISCUSSION

We have shown here that one *Mhc* class I allele is associated with antagonistic effects in terms of susceptibility/resistance to two malaria parasites. Although frequently evoked in theoretical models as a mechanism allowing the maintenance of alleles with deleterious effects (Zhang *et al.* 2002), empirical evidence for antagonistic pleiotropy involving two pathogens is scarce, especially for free ranging hosts and their natural parasites.

The maintenance of the allele *pado123* obviously depends on the costs and benefits associated with the presence of the allele, which in turn depend on the effect of *Plasmodium* and *Haemoproteus* parasites on their hosts as well as on parasite prevalence. If we were facing benign parasites there would be no benefit to be resistant and no cost to be susceptible. Infection by haematozoa has been reported to induce mortality in domestic birds, such as poultry and canaries (McCutchan *et al.*, 2004; Williams 2005) and empirical evidence has built up to show that both *Plasmodium* and *Haemoproteus* strains can have a substantial impact on their avian hosts. *Plasmodium* infection of native Hawaiian birds has become a text-book example of parasite-driven local extinction of hosts (VanRiper *et al.* 1986). Experimental infections of house sparrows with the *Plasmodium* SGS1 strain have also shown that infected hosts have increased anaemia compared with sham-infected sparrows and that the degree of anaemia positively correlates with parasite intensity (Guivier *et al.* unpublished work). Similarly, medication experiments have shown that *Haemoproteus* infection in Blue tits (*Cyanystes caeruleus*) and in House martins (*Delichon urbica*) has a detrimental effect on reproductive success and demographic variables (Merino *et al.* 2000; Marzal *et al.* 2005). Finally, the intensity of *Haemoproteus columbae* infection has been shown to correlate with juvenile survival in feral pigeons (*Columba livia*), highly infected individuals suffering from reduced survival (Sol *et al.* 2003). We found that *pado123*$^+$ and *pado123*$^-$ sparrows achieve similar fitness if the cost of the infection with the *Haemoproteus* strain is at least twice the cost induced by the *Plasmodium* strain. This is somehow contrary to the common belief that *Haemoproteus* parasites are less virulent than *Plasmodium* strains (Valkiûnas 2005). Unfortunately, however, we badly lack comparative studies that have assessed the relative impact of *Plasmodium* and *Haemoproteus* parasites on host fitness in the same host species.

An association between a *Mhc* class I allele and increased susceptibility to *Plasmodium* SGS1 has already been reported in another house sparrow population (Bonneaud *et al.* 2006). Interestingly, susceptibility alleles have been reported to occur almost as often as resistance alleles, both in humans and wildlife species (Segal & Hill 2003; Schad *et al.* 2005; Bonneaud *et al.* 2006). *Mhc* alleles with antagonistic effects on different pathogens have also been reported but only for lab strains of mice (B10) (Apanius *et al.* 1997). For instance, the allele H-2^b confers resistance to Sendai virus, *Toxoplasma gondii, Trichuris muris, Streptococcus pyogenes, Heligmosomoides polygyrus* and susceptibility to *Borrelia burgdorferi* and *Leishmania donovani* (Apanius *et al.* 1997 and references therein). Recently, a similar antagonistic effect has also been described in a plant-pathogen system (oat-fungus). Lorang *et al.* (2007) showed that the *LOV1* gene, a member of the NBS-LRR resistance gene family, confers susceptibility to the fungus *Cochliobolus victoriae*. NBS-LRR (nucleotide-binding site-leucine-rich repeats) proteins are defense proteins structurally similar to proteins of the innate immune response of animals.

To our knowledge, we show for the first time that these pleiotropic effects can also operate in natural animal host-parasite systems. Experimental infections with the two parasites, of course, are necessary to confirm this pattern. However, whereas *Plasmodium* strains can be transferred from one infected host to another, *Haemoproteus* parasites need to be passaged through the dipterean vector, which is unfortunately unknown. Nevertheless, experimental infections conducted in two other populations of house sparrows have confirmed the existence of susceptibility alleles to *Plasmodium* SGS1 parasites (Guivier *et al.* unpublished work).

An alternative interpretation for the positive association between specific *Mhc* alleles and parasite prevalence is that hosts not carrying the allele had succumbed to the infection and only 'resistant' birds carrying the allele have actually managed to survive by converting the infection to a low chronic level (Westerdahl *et al.* 2005). This is of course an interesting possibility that deserves to be addressed using an experimental approach.

Patterns of association between *Mhc* alleles and infectious diseases might also arise because of other genes inherited by related individuals. To check this hypothesis, we used microsatellite markers to assess the relatedness of *pado123*$^+$ sparrows and found no evidence in support to the hypothesis.

The precise molecular mechanisms through which these susceptibility alleles operate are largely unknown. Suggested mechanisms involve the failure to present pathogen epitopes to T-cells (Kast *et al.* 1986) or more intriguingly, the idea that MHC molecules could present pathogen epitopes that function as a decoy. TCR binding to these epitope/MHC molecule complexes might fail to elicit an effective parasite clearance (O'Brien & Carrington 1999).

Whatever the precise molecular mechanism that allows one parasite strain to take advantage of the presence of the allele, these results suggest that antagonistic effect of immune genes on different parasites can contribute to explain the persistence of susceptibility alleles, at least under certain conditions. Genes that affect multiple traits are thought to be common in nature (Otto 2004). Examples of genes with antagonistic effects concern haemoglobin-associated disease loci that appear to protect the carrier from infections with malaria parasites (Nagel 2001). Glucose-6-phosphate dehydrogenase (*G6PD*) enzyme deficiency is another classical example of a human genetic disorder that seems to have been positively selected in areas with high malaria prevalence (Tishkoff & Verrelli 2004). The worldwide distribution of G6PD deficiency show a tight correlation with risk of *Plasmodium* infection suggesting that failure in the enzyme might protect against malaria. Both *in vivo* and *in vitro* studies have confirmed the role played by G6PD deficiency in malaria protection, probably because G6PD deficient red blood cells infected with *Plasmodium* experience high oxidative stress that might result in cell phagocytosis and parasite mortality (Tishkoff & Verrelli 2004). The signature of balancing selection on genes that expose to enhanced risk of disease has also been, recently, found in a gene coding for the poliovirus receptor protein (*CD155*) (Suzuki 2006) and a genetic variant of the signal transducer and activator of transcription (*STAT6*) gene that is associated with increased susceptibility to asthma an protection from *Ascaris* worm infection (Peisong *et al.* 2004).

In addition, or alternatively to a direct effect of *pado123* on resistance/susceptibility to malaria parasites, we would like to stress that within-host competition between parasite strains could also explain the observed results. Parasites exploiting the same resources in the host are potentially involved in competitive interactions that may contribute to regulate the number of co-existing individuals (deRoode *et al.* 2005). Specific immune recognition of different parasite epitopes by MHC molecules can therefore alter the strength of the competitive interactions between parasites. If *pado123* confers a quantitative resistance towards *Haemoproteus*, and *Haemoproteus* and *Plasmodium* compete for resources within the host, *pado123* may reduce the within-host growth rate of *Haemoproteus* and consequently reduce the strength of the competition acting on *Plasmodium*. According to this scenario the observed positive association between *pado123* and *Plasmodium* might not be the direct consequence of the presence of the allele but instead the outcome of the altered competition with *Haemoproteus*. Although still anecdotic, the observed pattern of co-infections suggests a role for *pado123* in the relative within host growth of the two parasite strains. Among the 6

co-infected birds, those carrying the *pado123* allele ($n = 3$) were predominantly infected by the *Plasmodium* strain, whereas those not carrying the allele ($n = 3$) were predominantly infected by the *Haemoproteus* strain. Nevertheless, this evidence is indirect and based on the difference between the nested and the strain specific PCR. Direct quantification with smear counts or quantitative PCR is thus required. However, in line with this argument, Råberg *et al.* (2006) have recently shown that competition between two clones of *Plasmodium chabaudi* was affected by the immune status of the hosts (nude vs. T-cell-reconstituted nude mice).

To the best of our knowledge, the role played by *Mhc* genes in the process of parasite competition and the virulence of mixed infections has not been properly evaluated. MHC-mediated selection can alter the competitive equilibrium between parasite strains or species and affect the level of optimal virulence in mixed infections. A theoretical appraisal of such effect might provide fruitful insight on the expected direction of the evolutionary changes.

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