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Associations between malaria and MHC genes in a migratory songbird

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Malaria parasites are a widespread and species-rich group infecting many wild populations of mammals, birds and reptiles. Studies on humans have demonstrated that genetic factors play a key role in the susceptibility and outcome of malaria infections. Until the present study, it has not been examined whether genetic variation in hosts is important for the outcome of malaria infections in natural avian populations.

We investigated associations between major histocompatibility complex (MHC) genes and prevalence of three different avian malaria parasites (Haemoproteus payeri (GRW1), Plasmodium sp. (GRW2) and Plasmodium sp. (GRW4)) in a long-term study of great reed warblers Acrocephalus arundinaceus. We hypothesized that the MHC genes could either give full protection against a malaria infection, or confer protection against lethal malaria and direct the infection towards being milder. We found a positive association between numbers of MHC class I alleles (a measure of level of heterozygosity) and prevalence of the GRW2 parasite, suggesting the latter scenario. There was also a positive association between a specific MHC allele (B4b), previously shown to be under frequency-dependent selection in the study population, and prevalence of GRW2. These associations suggest that individuals carrying either a large number of MHC alleles or a specific MHC allele are protected against lethal malaria infections.

Keywords: major histocompatibility complex; malaria parasites; lethal infection; genetic diversity; passerine; bird

1. INTRODUCTION

The major histocompatibility complex (MHC) contains the most variable genes known in vertebrates (Klein 1986). This remarkable variation is believed to be maintained by balancing selection, where the major selective force is different parasites and pathogens interacting with their hosts (Jeffery & Bangham 2000; Hess & Edwards 2002). Each MHC molecule binds and presents a limited number of peptides to T-cells, and when the bound peptide is non-self, an immune reaction will follow (Hughes & Yeager 1998). Therefore, according to one theory, individuals being overall heterozygous at the MHC loci should have the best protection against diseases as they express the largest repertoire of MHC molecules (Doherty & Zinkernagel 1975a,b; Nei & Hughes 1991).

MHC heterozygote advantage has been reported for two human diseases, hepatitis B and HIV-1 (Thursz et al. 1997; Carrington et al. 1999), and for Salmonella infections in MHC-congenic mice strains (Penn et al. 2002; McClelland et al. 2003). In natural populations of threespined sticklebacks (Gasterosteus aculeatus), an optimal number of MHC alleles (a measure of heterozygosity) have been shown to reduce the parasite load (Wegner et al. 2003).

MHC allelic diversity may not be selected for directly, but can result from negative frequency-dependent selection (Bodmer 1972). Allelic diversity will be maintained if different MHC alleles give protection against different parasites, and the composition of the parasite community varies on a temporal and/or spatial scale (Hedrick 2002). Direct associations between certain MHC alleles and resistance to specific diseases have been found in several studies on humans, on chickens (Gallus gallus domesticus), in mouse (Mus musculus) strains (Briles et al. 1977; Hormaeche et al. 1985; Nauciel et al. 1988; Hill et al. 1991; Plachy et al. 1992; Hill 2001), in studies from a hatchery population of Atlantic salmon (Salmo salar; Langefors et al. 2001; Lohm et al. 2002) and in an unmanaged Soay sheep (Ovis aries) population (Paterson et al. 1998). To our knowledge, however, there are no studies of specific MHC alleles and disease resistance in strictly natural and free-ranging avian populations.

Avian malaria parasites, comprising species in the genera Plasmodium and Haemoproteus, have achieved considerable attention (Bensch et al. 2000; Ricklefs & Fallon 2002; Waldenström et al. 2002; Bensch & Åkesson 2003; Fallon et al. 2003). This species-rich group of vector-borne blood parasites is taxonomically related to the parasites causing malaria in humans, but are restricted to bird hosts. In avian malaria, as in human malaria (Hill et al. 1991), it is the first exposure to the infection that may cause the severest fitness consequences (i.e. the acute infection; Atkinson & van Riper 1991). On the other hand, chronic stages of malaria infections are characterized by having no or only mild fitness effects (Atkinson & van Riper 1991; Hill et al. 1991). Once an individual has been infected with malaria, the infection may persist for years or...

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even a lifetime (Atkinson & van Riper 1991). In humans, it has been shown that genetic factors play a key role for the susceptibility and outcome of the malaria disease (Fortin et al. 2002).

When interpreting patterns of associations between prevalence of malaria infection (the presence of parasites in the blood stream of a host) and host MHC genotype, it is essential to consider when the sampling of the study population is carried out relative to the general timing of the acute infections. In a study of human children, Hill et al. 1991 found that specific MHC classes I and II alleles gave protection against the development of severe malaria. The entire course of the disease was registered and it was possible to follow how the surviving and non-surviving individuals responded to the infection. In natural populations of birds, however, individuals that are severely ill will rarely be caught and sampled. Thus, data collected from wild birds will mainly contain two groups of birds: (i) those showing no malaria parasites, implying either that they have not been infected or that they have completely cleared an infection and (ii) those showing (often low levels of) malaria infection, implying that they have survived the acute infection and now are in the chronic and probably rather harmless stage of a malaria infection. This means that when adults in natural populations are sampled (i.e. when they potentially already have been exposed to the acute phase of the malaria infection), the most probable expectation is that birds scored as infected are those that have managed to withstand the severe effects of malaria.

We have conducted a long-term field study of a great reed warbler (Acrocephalus arundinaceus) population in Sweden. Earlier genetic studies have shown that the great reed warbler has a very variable MHC (Westerdahl et al. 1999, 2000, 2004b) and that it is advantageous to be heterozygous at MHC class I loci, because such individuals tend to have higher survival (Hansson et al. 2004). Specifically, we found that one MHC class I allele (B4b) varied significantly in frequency between years among cohorts in the breeding population (Westerdahl et al. 2004a). In parallel with the MHC screening, PCR-based detection and identification of avian malarial parasites have been performed. The relative frequency of the three most common parasite lineages (or rather parasite species since they all have diverged mitochondria and nuclear sequences; GRW1, Haemoproteus payeskyi, GRW2, Plasmodium sp. and GRW4, Plasmodium sp.; Bensch et al. 2004, unpublished work) also show considerable variation in prevalence between years. Thus, the temporal variation of the malaria parasites could possibly cause the temporal variation of the MHC alleles by exerting different selection pressures in different years.

The great reed warbler is a long-distance migrant and will therefore be exposed to the avian malaria parasite faunas of both Europe and Africa. These parasite faunas differ substantially; interestingly, recent studies have indicated that parasite lineages that normally occur in resident African birds can also be transmitted to migrant European warbler species when wintering in Africa (Bensch et al. 2000; Waldenström et al. 2002). Furthermore, these parasites should have evolved to peak in parasitemia with sexual life stages (acute infection and relapses) at the times when there are competent vectors in the environment. The three malaria lineages GRW1, GRW2 and GRW4 are probably transmitted to the great reed warblers in Africa (Waldenström et al. 2002).

In the present study of breeding great reed warblers, we investigate whether overall heterozygosity at MHC class I loci is associated with prevalence of malaria infection, and also if the MHC class I allele, B4b, that previously was found to vary significantly among cohorts, is associated with the occurrence of any of the three most common malaria lineages (GRW1, GRW2 and GRW4). We predict that: (i) if there are MHC alleles that give full protection against all stages of malaria infection, then there will be a negative association between number of MHC alleles and prevalence of malaria infection. (2) However, if the MHC alleles only confer protection against lethal malaria and direct the infection towards being mild or chronic, then there will be a positive association between number of MHC alleles and prevalence of malaria parasites.

2. MATERIAL AND METHODS

(a) Study species

The great reed warbler is a medium-sized passerine bird that breeds in reed marshes in Europe and Asia, and winters in tropical Africa (Cramp 1992). Since 1984, we have conducted detailed studies of the breeding ecology of an entire great reed warbler population at Lake Kvismaren, in south Central Sweden (59°10’ N, 15°25’ E; Bensch 1996; Hasselquist 1998). The majority (95–100%) of the breeding birds and their nestlings have been ringed, each with an aluminium ring and an individual-specific combination of colour rings, and blood samples have been collected. A few birds founded the population in 1978, and from the late 1980s, the breeding population reached a fairly stable level of about 60 breeding adults (Bensch & Hasselquist 1991; Hansson et al. 2000).

In the present study, we investigated 340 breeding great reed warblers in Lake Kvismaren that belonged to cohorts hatched between 1983 and 1996. All birds included have survived until at least 1 year old, which means that they have been exposed to malaria parasite faunas of both Africa and Europe.

(b) Screening of MHC class I variation

The MHC class I alleles in great reed warblers have not been possible to assign to specific loci, probably because of homogenization of alleles across loci (Westerdahl et al. 1999, 2000b). We therefore studied the MHC class I variation using sequence-specific amplification of exon 3 sequences (encoding the peptide-binding region of the MHC molecule) from several MHC genes simultaneously. In order to preferentially amplify transcribed alleles, we used cDNA sequences as references when designing exon 3 primers (HN36 and HN38; Westerdahl et al. 1999, 2000b). The primers HN36 and HN38 have different motifs in their 3′ end (both motifs are found at positions 12 to 14 in exon 3). Additional 3′-end motifs were found in the exon 3 sequences; however, we chose not to use these motifs because they would have amplified alleles that were either non-variable or found in several pseudogene sequences. Using this method of amplification of exon 3 sequences, we amplify a certain range of alleles, where the majority of the alleles are transcribed. It shall be noted that all class I alleles are not amplified.
The polymerase chain reaction (PCR) products from the two different primer combinations (HN36/HN38 and GC46; Westerdahl et al. 2004b) were separated by the DGGE method (denaturant gradient gel electrophoresis; Myers et al. 1987). This screening method identifies 2–12 exon 3 sequences per individual (for simplicity, we here mention these sequences as ‘MHC alleles’, although we are aware that they stem from several different loci rather than a single specific locus). We do not suggest that the number of transcribed class I loci varies between one and six (all individuals being heterozygote at all loci) in great reed warblers, but rather that the individuals which are screened as having two or three alleles (n = 5) carry fewer alleles than individuals that are screened as having five alleles, for example. Furthermore, the DGGE method does not have a 100% resolution and therefore we could have failed to separate some alleles. The total number of MHC alleles is a rather good estimate of MHC heterozygosity since individuals being heterozygote at all loci) in great reed warblers, but rather that the individuals which are screened as having two or three alleles (n = 5) carry fewer alleles than individuals that are screened as having five alleles, for example. Furthermore, the DGGE method does not have a 100% resolution and therefore we could have failed to separate some alleles. The total number of MHC alleles is a rather good estimate of MHC heterozygosity since individuals scored as having few alleles are homozygous at more loci than an individual with several alleles (Westerdahl et al. 2004b). A subset of the exon 3 sequences has been sequenced and the screening method (using motif-specific PCR and DGGE) is highly repeatable (Westerdahl et al. 2004b).

(c) Screening of microsatellite variation
To obtain an average estimate of the heterozygosity in the entire great reed warbler genome, 340 individuals were typed for allelic variation at 18 microsatellite loci: Aar2-5, Aar8 (Hansson et al. 2000); Ppi2 (Martinez et al. 1999); Ase7, Ase9, Ase11, Ase18, Ase34, Ase42, Ase44, Ase58, Ase60 (Richardson et al. 2000); Ase15 (D. S. Richardson, unpublished work; see Hansson et al. 2004); Hru5 (Primmer et al. 1996); and Sjr4 (D. B. McDonalds & W. K. Potts, unpublished work; see Hansson et al. 2000). Primer sequences and amplification conditions are given in Hansson et al. (2000, 2004). Multilocus heterozygosity was calculated as the number of heterozygotic loci (scored ‘1’ for each heterozygous locus and ‘0’ for each homozygous locus) divided by the number of loci examined (i.e. 18 loci).

(d) Screening of avian malarial infections
Several great reed warblers survived in the study population for more than 1 year and they were screened for malaria infection in all years from which we had obtained a blood sample. In the present analyses, we scored individuals to be infected if they had been typed positive for the lineages GRW1, GRW2 or GRW4 at least once during their life. Using a nested PCR approach (Waldenström et al. 2004), a 480 bp long fragment of the malaria parasite cytochrome b gene was amplified from blood samples of infected great reed warblers. This method is highly repeatable, and has a detection limit as low as one infected red blood cell per 100 000 (Waldenström et al. 2004). On a few occasions (n = 9), two parasites infected the same host. The PCR products of these double infected individuals were cloned and several clones were sequenced to reveal the sequences of the concordant infections (Waldenström et al. 2004).

(e) Statistical analyses
We analysed the overall genetic differentiation between infected and non-infected recruiting great reed warblers based on 23 MHC alleles. This was done separately for the three malaria infections, GRW1, GRW2 and GRW4, by a genetic structure analysis (AMOVA) implemented in ARLEQUIN 2.000 (Schneider et al. 2000). The MHC alleles were treated as either present (1) or absent (0). We then analysed presence or absence of malaria infection among recruited great reed warblers (dependent variable) with number of MHC bands or multilocus microsatellite heterozygosity as the independent variable using logistic regressions (SAS 8.02; Genmod module, Logit link function, binomial error distribution, Type 3 option (SAS 1990)). Because the scale parameters (deviance/d.f.) of all models were less than 1 (indicating under-dispersion), the significance of parameters was tested with a χ²-test (SAS 1990; Crawley 1993). Finally, we ran a χ²-test for association between specific MHC alleles and malaria infections and also Spearman rank correlation (r_{s}) in SYSTAT 9.0 (Wilkinson 1998). To visualize the change in proportion of individuals with malaria infection GRW2 against number of MHC class I alleles, we used the cubic spline method in a DOS-version of the cubic spline program by Schluter (1988). All tests are two-tailed.

3. RESULTS
The mean prevalence of the three most common malaria lineages in breeding great reed warblers at our study site were 23% (GRW1), 8% (GRW2) and 20% (GRW4), and the prevalence varied considerably between cohorts (figure 1).

Individuals infected or non-infected with malaria parasite GRW2 tended to be genetically differentiated in terms of MHC alleles (23 MHC alleles, AMOVA 1,339, F_{s} = 0.013, p = 0.040), but this was not at all the case for infection with malaria parasite GRW1 (AMOVA 1,339, F_{s} = 0.0019, p = 0.23) and GRW4 (AMOVA 2,339, F_{s} = 0.001, p = 0.66).

The average number of MHC alleles was significantly higher in individuals infected with GRW2 (7.1 ± 1.4 (s.d.), n = 26) compared with uninfected individuals (6.3 ± 1.8 (s.d.), n = 314; U = 2963, p = 0.018, figure 2). The prevalence of malaria infection GRW2 was significantly positively associated with the number of MHC alleles, an estimate of MHC heterozygosity (logistic regression, n = 340, χ² = 7.17, p = 0.0074; table 1), demonstrating that the there is a nonlinear relationship between the number of MHC alleles and the prevalence of GRW2. This nonlinear relationship is visualized in a cubic spline plot (figure 3). We cannot tell from our results whether the prevalence of GRW2 is highest among birds having an intermediate number of MHC alleles, because of the very large 95% confidence interval of the cubic spline function at greater than eight MHC alleles (figures 2 and 3).

The most parsimonious conclusion is therefore that the prevalence of GRW2 reached a plateau at eight MHC alleles where after a further increase in alleles has no effect on the prevalence (figure 3). In contrast, there was no correlation between either infection with GRW1 (logistic regression, n = 340, χ² = 0.38, p = 0.54), or infection with GRW4 (logistic regression, n = 340, χ² = 0.033, p = 0.85) and the number of MHC alleles.

The prevalence of GRW2 was not correlated with genome-wide heterozygosity, measured at 18 microsatellite loci (logistic regression, n = 340, χ² = 0.40, p = 0.53). There was no correlation between infections

with either GRW1 (logistic regression, \( n = 340, \chi^2 < 0.01, p = 1.00 \)) or GRW4 (logistic regression, \( n = 340, \chi^2 = 0.65, p = 0.42 \)) and microsatellite heterozygosity. Finally, there was no correlation between number of MHC alleles and microsatellite heterozygosity (\( r_s = 0.039, n = 340, p = 0.38 \)).

There was a significant positive association between the MHC class I allele B4b (this allele has been shown to vary significantly between years among cohorts of great reed warblers; Westerdahl et al. 2004a) and infection with GRW2 (table 2). There were no significant associations between the other two parasites and allele B4b (table 2). Afterwards, we tested whether there were associations between any of the remaining 22 MHC alleles and infection with GRW1, GRW2 and GRW4, but found no such correlations (\( p > 0.03 \) in all cases, hence, they were far from significant after Bonferroni correction).

There was a positive correlation between allele B4b and the number of MHC alleles (logistic regression, \( n = 340, p = 0.0074 \)).

Table 1. Results from logistic regression analyses of the variation in prevalence of the malaria parasite GRW2 in relation to number of MHC alleles (MHCalleles) and MHC allele B4b in great reed warblers. Parameters entered the models at \( p < 0.1 \).

<table>
<thead>
<tr>
<th>parameter</th>
<th>( n_{\text{individuals}} )</th>
<th>estimate</th>
<th>( \chi^2 )</th>
<th>( p^a )</th>
</tr>
</thead>
<tbody>
<tr>
<td>MHCalleles</td>
<td>340</td>
<td>2.60</td>
<td>7.17</td>
<td>0.0074</td>
</tr>
<tr>
<td>MHCalleles × MHCalleles</td>
<td>-0.17</td>
<td>5.82</td>
<td>0.0159</td>
<td></td>
</tr>
<tr>
<td>MHCalleles</td>
<td>284(^b)</td>
<td>4.26</td>
<td>8.48</td>
<td>0.0036</td>
</tr>
<tr>
<td>MHCalleles × MHCalleles</td>
<td>-0.30</td>
<td>7.74</td>
<td>0.0054</td>
<td></td>
</tr>
<tr>
<td>MHCalleles</td>
<td>340</td>
<td>-0.16</td>
<td>5.19</td>
<td>0.0227</td>
</tr>
<tr>
<td>MHCalleles × MHCalleles</td>
<td>0.87</td>
<td>3.46</td>
<td>0.0629</td>
<td></td>
</tr>
<tr>
<td>allele B4b</td>
<td></td>
<td>0.95</td>
<td>4.09</td>
<td>0.0432</td>
</tr>
</tbody>
</table>

\(^a\) d.f. = 1 in all cases.
\(^b\) Individuals that have allele B4b have been excluded.
study so far has indicated that carrying very few MHC alleles could be advantageous, whereas several studies have implied that having an average number of MHC alleles incurs an advantage (Wegner et al. 2003; Kurtz et al. 2004).

A number of studies from wild bird populations have shown that there are severe fitness costs associated with the acute phase of avian malaria (Richner et al. 1995; Oppliger et al. 1996; Atkinson et al. 2001; Sol et al. 2003). In the great tit (Parus major), females with Plasmodium malaria infections lay smaller egg clutches (Richner et al. 1995; Oppliger et al. 1996). In feral pigeons (Columba livia), young birds that were infected with Haemoproteus columbae had a lower probability of surviving until adulthood compared with uninfected birds (Sol et al. 2003). In Hawaii, Plasmodium relictum is one of the primary factors responsible for the disappearance of Hawaiian Honeycreepers (Drepanidinae spp.; Atkinson et al. 2001). However, the parasite P. relictum was recently introduced to Hawaii, and the high sensitivity of the native birds to this parasite might be because they may not have had time to evolve an appropriate immune response against it. Hayworth et al. (1987) found that under laboratory conditions, canaries (Serinus Canaria) infected with P. relictum had a reduced ability to thermoregulate at the peak of the infection, suggesting a fitness cost. It is clear, however, that, Plasmodium parasites of the same strain may differ in pathogenicity in different avian species, although Plasmodium infections are very likely to cause deaths in wild birds (reviewed in van Riper et al. 1994).

The acute phase of the malaria infection has not been studied in the great reed warbler since it usually occurs when the naive birds spend their first winter in Africa. In the present study, we have sampled the recruiting birds at their breeding site when the malaria infection probably has reached its chronic state. Hence, the parasites are detectable with the PCR technique, but the infected birds show no symptoms (Atkinson & van Riper 1991; Bruce et al. 2000). The intensity of the H. payevskyi (GRW1) malaria infection in blood smears (measured as number of blood parasites per 10,000 erythrocytes; Waldenström et al. 2004) is highest in the spring when the great reed warblers are newly arrived from Africa and then successively decreases over the summer (D. Hasselquist, unpublished work). The intensity of Plasmodium infections in blood smears is more difficult to investigate because these parasites often occur at very low densities (van Riper et al. 1994). In the great reed warblers, 0.6% of 319 screened blood smears of breeding adults had erythrocytes infected with GRW2 parasites. In the same samples, the PCR-based screening detected at least 4% prevalence (D. Hasselquist, unpublished work). Therefore, the intensity of the GRW2 infection must generally be very low when the great reed warbler recruits return to their breeding site. This also strengthens the notion that this malarial parasite is transmitted in Africa, as parasitemia in blood should peak when suitable vectors occur.

The overall prevalence of Haemoproteus and Plasmodium infections among breeding great reed warblers at our study site is 43% and, as expected, there are no detectable fitness costs associated with being infected (S. Bensch, unpublished work). This pattern also holds in separate analyses for the three lineages GRW1, GRW2 and GRW4.
(S. Bensch, unpublished work). One probable explanation is, as mentioned above, that the acute phase of the malaria infection occurs at the great reed warbler’s wintering grounds in Africa. The recruiting individuals that carry malaria infections to the European breeding grounds are then those that have survived the infection transmitted in Africa. It is well known that avian malaria parasites often infect birds in their first year of life (van Riper et al. 1994) and we found a similar pattern; juveniles have a 6% probability of becoming infected with GRW2 during their first winter while older birds have a 2.5% probability of becoming infected (S. Bensch, unpublished work). Once an individual has been infected with malaria, the infection may persist for years or even a lifetime (Atkinson & van Riper 1991). In the great reed warbler, there is a 50% probability that an individual that is infected with malaria (GRW1, GRW2 or GRW4) one year also will carry the same infection the following year (S. Bensch, unpublished work).

Several human studies have found associations between certain MHC alleles and resistance to malaria (Jepson et al. 1997; Hill 2001; Flori et al. 2003), and Hill et al. (1991) have shown that such MHC alleles were under selection from the malaria parasites. In the great reed warbler, there was a positive association between the MHC class I allele B4b and the malaria infection GRW2 (tables 1 and 2). We have previously shown that this specific allele (B4b) varied significantly in frequency among breeding birds belonging to different cohorts, presumably resulting from a changing community of pathogens (Westerdahl et al. 2004a). Furthermore, the prevalence of malaria infection GRW2 varies considerably between cohorts in our study population (figure 1). Our results thus imply that the MHC allele B4b is under selection from one or several pathogens (Westerdahl et al. 2004a), and that one of these pathogens is probably GRW2.

In the present study, we have used a screening method that is based on the amplification and separation of transcribed (expressed) MHC class I alleles. We have used a protocol that constrains the amplification to a limited set of expressed alleles in order to avoid screening of non-functional genes. Because of this selective approach, we fail to amplify some alleles that are found at expressed loci. For example, we found one individual that was screened to have two alleles, and it is possible that we have missed one or two alleles as a result of our protocol, but certainly not a large number of alleles. Hence, our method allows us to rank individuals for expressed MHC heterozygosity, even though we cannot (using this method) estimate the exact number of expressed MHC alleles in each individual. Using the present screening method, individuals that have a large number of alleles are more heterozygote than individuals that are screened as having few alleles.

The overall genetic differentiation of MHC alleles, the number of MHC alleles and the squared number of MHC alleles all contributed significantly to explain prevalence of malaria infection GRW2 (table 1). Relating GRW2 to the number of MHC alleles using a cubic spline function suggests that there is a positive relationship between prevalence of GRW2 and the number of MHC alleles, and also that this correlation levels off at about seven MHC alleles (figure 3). The 95% confidence interval of the cubic spline function becomes large for values of alleles greater than eight and it requires further studies before we can separate whether there is an optimal number of alleles, as suggested in studies of sticklebacks (Wegner et al. 2003), or whether the function has reached a plateau. Theoretical studies predict that there are an optimal number of MHC genes (loci; Nowak et al. 1992; Takahata 1995). Taken together, our results imply that carrying six or more MHC alleles seems advantageous for surviving the acute malaria infection and individuals with a large number of MHC alleles more often carry advantageous MHC alleles, one being allele B4b.

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