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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 payevskyi* (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 1975*a*,*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 three-spined 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, 2004*b*) 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.* 2004*a*). 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 payevskyi*, 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, 2004*b*). 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, 2004*b*). 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.* 2004*b*) 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 scored as having few alleles are homozygous at more loci than an individual with several alleles (Westerdahl *et al.* 2004*b*). 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.* 2004*b*).

(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 heterozygous 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 identifying parasitemia 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 χ^2-test (SAS 1990; Crawley 1993). Finally, we ran a χ^2-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, $AMOVA_{1,339}$, $F_{st}=0.013$, $p=0.040$), but this was not at all the case for infection with malaria parasite GRW1 ($AMOVA_{1,339}$, $F_{st}=0.0019$, $p=0.23$) and GRW4 ($AMOVA_{2,339}$, $F_{st}=0.001$, $p=0.66$).

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, $n=340$, $\chi^2=7.17$, $p=0.0074$; table 1). Furthermore, the squared number of MHC alleles contributed significantly to the model (logistic regression, $n=340$, $\chi^2=5.82$, $p=0.016$; 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, $n=340$, $\chi^2_1=0.38$, $p=0.54$), or infection with GRW4 (logistic regression, $n=340$, $\chi^2_1=0.033$, $p=0.85$) and the number of MHC alleles.

The prevalence of GRW2 was not correlated with genome-wide heterozygosity, measured at 18 microsatellite loci (logistic regression, $n=340$, $\chi^2_1=0.40$, $p=0.53$). There was no correlation between infections

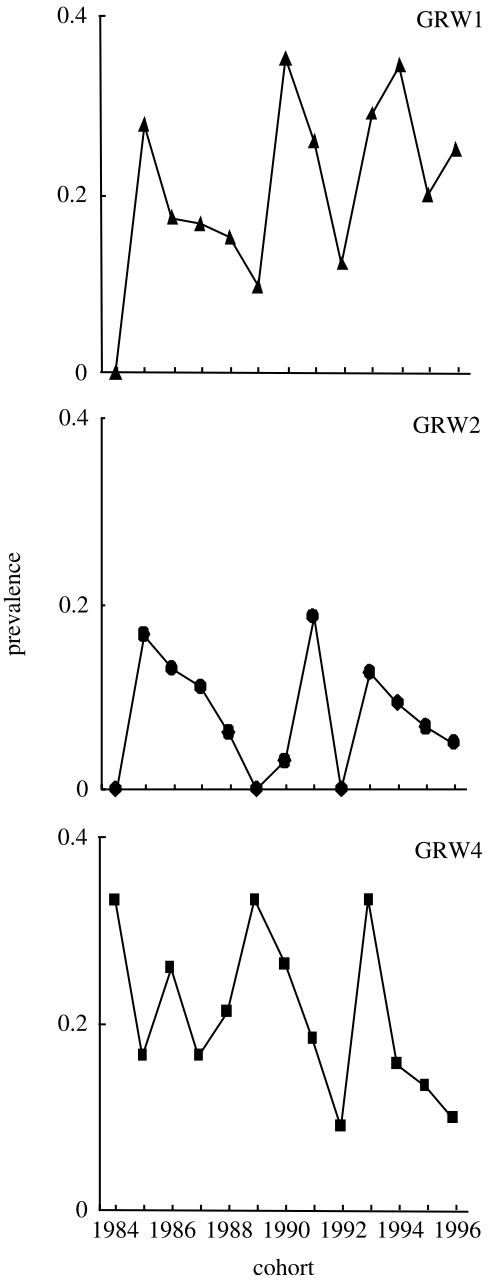

Figure 1. Prevalence of malaria infection GRW1, GRW2 and GRW4 in 13 successive cohorts of great reed warblers breeding at Lake Kvismaren (cohort, number of individuals: 1983, 1 (not shown); 1984, 6; 1985, 16; 1986, 23; 1987, 18; 1988, 33; 1989, 21; 1990, 34; 1991, 27; 1992, 33; 1993, 24; 1994, 32; 1995, 30; 1996, 40).

with either GRW1 (logistic regression, $n=340$, $\chi^2_1<0.01$, $p=1.00$) or GRW4 (logistic regression, $n=340$, $\chi^2_1=0.65$, $p=0.42$) 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.* 2004*a*) 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, $n=340$,

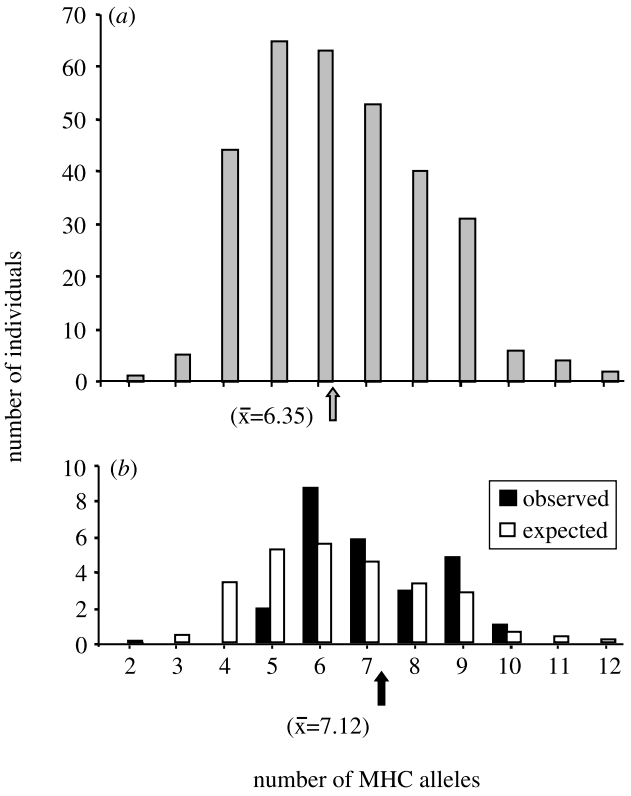

Figure 2. The distribution of number of MHC alleles (an estimate of heterozygosity) in great reed warblers, which are either (*a*) uninfected (grey bars) or (*b*) infected (black bars) with malaria infection GRW2. The distribution of MHC alleles in individuals that are expected to be infected with malaria infection GRW2 (8% of the total numbers of individuals are known to be infected with GRW2) is obtained directly from the distribution in (*a*), and are indicated with open bars (*b*). The average number of MHC alleles in infected (black arrow) and uninfected individuals (grey arrow) are shown.

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

parameter	$n_{individuals}$	estimate	χ^2	p[a]
$MHC_{alleles}$	340	2.60	7.17	0.0074
$MHC_{alleles}\times MHC_{alleles}$		−0.17	5.82	0.0159
$MHC_{alleles}$	284[b]	4.26	8.48	0.0036
$MHC_{alleles}\times MHC_{alleles}$		−0.30	7.74	0.0054
$MHC_{alleles}$	340	2.52	6.18	0.0129
$MHC_{alleles}\times MHC_{alleles}$		−0.16	5.19	0.0227
allele B4b		0.87	3.46	0.0629
$MHC_{alleles}$	340	−0.19	2.76	0.0967
allele B4b		−0.95	4.09	0.0432

[a] d.f. = 1 in all cases.
[b] Individuals that have allele B4b have been excluded.

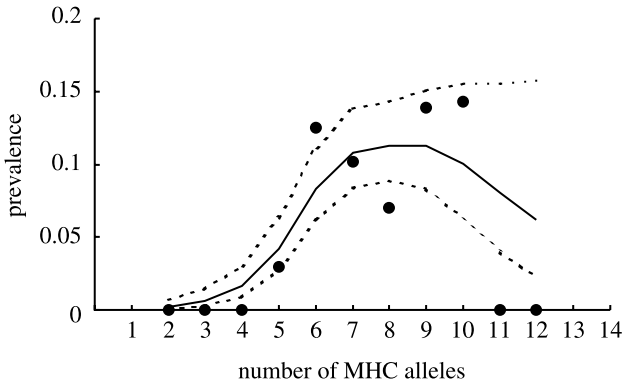

Figure 3. Cubic spline function that relates the prevalence of infection GRW2 with the number of MHC alleles (smallest value of cross-validation score, $\lambda = -1.00$). Mean (solid line) and one standard error of the predicted mean values (dotted lines) are shown.

Table 2. Malaria parasite prevalence (parasite GRW1, GRW2 and GRW4, respectively) in great reed warblers with and without MHC allele B4b.

parasite	prevalence (%) in individuals with B4b ($n=56$)	prevalence (%) in individuals without B4b ($n=284$)	χ^2	p
GRW1	29	21	1.34	0.25
GRW2	16	6	6.74	0.009
GRW4	16	20	0.56	0.45

$\chi^2_1 = 11.59$, $p = 0.0007$). Thus, individuals with many MHC alleles were more likely to carry the B4b allele. However, the correlation between MHC alleles and prevalence of GRW2 remained significant also after excluding all individuals carrying allele B4b (table 1). Finally, we included both the B4b allele and number of MHC alleles in a multiple logistic regression analysis, and found that both these factors appear to contribute to the model (table 1).

4. DISCUSSION

We found a positive association between numbers of MHC class I alleles and prevalence of the GRW2 malaria parasite among breeding great reed warblers in our study population (figure 2, table 1). This correlation was not explained by inbreeding, because birds with a large number of MHC alleles were not more heterozygous (measured at 18 microsatellite loci) than birds with few MHC alleles. Furthermore, there was also a positive association between MHC allele B4b and the prevalence of the GRW2 malaria parasite (table 2). These associations probably suggest that individuals carrying either a large number of MHC alleles or allele B4b survive infections of GRW2 more often than other individuals do. The large number of MHC alleles, or the allele B4b, does not give full protection against the GRW2 malaria parasite as such, but seems to confer protection against the lethal effects of the GRW2 infection. An alternative explanation to these associations is that individuals that carry a large number of MHC alleles, or carry allele B4b, are more prone to become infected with GRW2. However, we find this alternative explanation less probable since no 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 will usually occur 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 occurs.

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.* 2004*a*). 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.* 2004*a*), 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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