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MHC diversity, malaria and lifetime reproductive success in collared flycatchers

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Abstract

Major histocompatibility complex (MHC) genes encode proteins involved in the recognition of parasite-derived antigens. Their extreme polymorphism is presumed to be driven by co-evolution with parasites. Host–parasite co-evolution was also hypothesized to optimize within-individual MHC diversity at the intermediate level. Here, we use unique data on lifetime reproductive success (LRS) of female collared flycatchers to test whether LRS is associated with within-individual MHC class II diversity. We also examined the association between MHC and infection with avian malaria. Using 454 sequencing, we found that individual flycatchers carry between 3 and 23 functional MHC class II B alleles. Predictions of the optimality hypothesis were not confirmed by our data as the prevalence of blood parasites decreased with functional MHC diversity. Furthermore, we did not find evidence for an association between MHC diversity and LRS.

Keywords: fitness, genetic variation, immune response, major histocompatibility complex, optimality hypothesis, parasites, selection

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Introduction

Major histocompatibility complex (MHC) genes encode proteins involved in discrimination between self and nonself, a precondition for effective defence against parasites (Janeway *et al.* 2004). These genes show extremely high level of polymorphism, thought to be an effect of evolutionary response to a variety of fast-evolving parasites (Apanius *et al.* 1997; Bernatchez & Landry 2003). Mutations in parasites that prevent antigen recognition by the host MHC molecules will be strongly selected for; consequently, MHC types that restore parasite recognition will be favoured. Indeed, the evolution of MHC sequences appears to be driven by antigen-binding function of the molecules they encode. Polymorphisms are usually clustered at amino acid sites involved in interactions with antigens; these antigen-binding sites (ABS) typically show a marked excess of nonsynonymous (over synonymous) substitutions (reviewed in Bernatchez & Landry 2003; Garrigan & Hedrick 2003; Piertney & Oliver 2006). Further support for the role of parasites in promoting MHC polymorphism is provided by the growing evidence for the association among MHC types and susceptibility to disease (Hill *et al.* 1991; Kaufman & Wallny 1996; Thursz *et al.* 1997; Langefors *et al.* 2001; Froeschke & Sommer 2005; Eizaguirre *et al.* 2009; Kloch *et al.* 2010). However, with few exceptions (e.g. Paterson *et al.* 1998; Eizaguirre *et al.* 2009), fitness consequences of such associations are poorly understood.

Among birds, associations between MHC and malaria have been demonstrated (Bonneaud *et al.* 2006; Loiseau *et al.* 2008, 2011). Avian blood parasites belong to one of the three protist genera, *Haemoproteus, Leukocytozoon* or *Plasmodium*, and are transmitted via dipteran vectors (Valkiunas 2005). They cause symptoms similar to human malaria and can be highly virulent in naïve bird

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hosts (Atkinson & van Riper 1991, Atkinson *et al.* 2000). Even chronic infections were shown experimentally to exert a significant selection pressure on their hosts, by decreasing survival (La Puente *et al.* 2010) and reproductive success (Marzal *et al.* 2005; Asghar *et al.* 2011) of the parents, as well as by having detrimental effects on their offspring (Merino *et al.* 2000; Knowles *et al.* 2010).

Parasites are expected to adapt easily to the most common host MHC alleles; therefore, rare MHC alleles may gain an advantage (Bodmer 1972; Borghans *et al.* 2004). Such frequency-dependent selection may maintain high levels of MHC polymorphism in natural populations. Furthermore, parasite composition may change in time, favouring different MHC alleles at different time points (Hedrick 2002), or in space (Kloch *et al.* 2010; Loiseau *et al.* 2011). Another mechanism that may result in the maintenance of polymorphism in MHC genes is the heterozygote advantage resulting from an increased range of antigen types that may be presented by MHC heterozygotes (Doherty & Zingernagel 1975; Takahata & Nei 1990). By the same argument, possessing more MHC loci within the individual genome should allow more effective parasite recognition. Alternatively, too much MHC diversity may limit the repertoire of T lymphocyte variants produced by an individual as a result of extensive negative selection (Vidovic & Matzinger 1988). Extensive negative selection may compromise immune response, and theoretical models suggest that within-individual MHC diversity will be optimized rather than maximized (Nowak *et al.* 1992; Woelfing *et al.* 2009).

The optimality hypothesis can be tested in species showing interindividual variation in the number of expressed MHC loci, but this has only been performed in a few cases. In sticklebacks, water pythons and bank voles, an intermediate number of alleles have been shown to be associated with the lowest parasite load (Wegner *et al.* 2003a,b; Madsen & Ujvari 2006; Kloch *et al.* 2010). Fitness effects of intraindividual MHC diversity are even less explored. Bonneaud *et al.* (2004) found that house sparrow females with intermediate numbers of alleles laid the highest number of eggs in first clutches and that their nestlings survived better when compared to those of females with extreme numbers of alleles. In the most thorough study to date, Kalbe *et al.* (2009) found that, under semi-natural conditions, lifetime reproductive success (LRS) of three-spined sticklebacks is the highest when they carry intermediate number of MHC alleles. Here, we combine a large data set on LRS of female collared flycatchers (*Ficedula albicollis*) with data on malarial infection to test the prediction of the optimality hypothesis that prevalence of malaria reaches a minimum and LRS a concomitant maximum, at an intermediate level of within-individual MHC diversity.

Recent advances in typing of MHC profiles consisting of many coamplifying loci, based on new generation sequencing (Babik *et al.* 2009; Galan *et al.* 2010; Kloch *et al.* 2010; Zagalska-Neubauer *et al.* 2010), make it possible to test the optimality hypothesis in species showing a wide range of individual MHC diversity. Here, we take advantage of considerable interindividual variation in the number of functional MHC IIB genes in collared flycatchers (Zagalska-Neubauer *et al.* 2010) to test the effect of individual MHC diversity on malaria infection and on LRS. Some alleles may have very similar binding properties, so the number of alleles carried by an individual may not accurately reflect its antigen-binding range. Therefore, we do not only count alleles within individuals, but we also assess their functional diversity. The measure of functional diversity is based on physicochemical properties of amino acid residues known to be under positive selection, most likely resulting from their involvement in antigen binding.

Materials and methods

Data were collected in the population of collared flycatchers inhabiting southern Gotland (Swedish island on the Baltic Sea) in 2003. The collared flycatcher inhabits mainly deciduous and mixed forests in south-eastern Europe, and the range of this species expands towards north, probably due to climatic change. Collared Flycatchers first appeared on Gotland over a hundred years ago. Now, the population is relatively numerous and stable (the population size is estimated at 4000–5000 pairs) but remains isolated geographically from the main species range by *c.* 700 km distance because of the Baltic Sea. The collared flycatcher is a cavity-nesting bird species and breeds readily in nest boxes when provided. Our population inhabits nine plots of deciduous forests and one plot of coniferous forest separated from each other by arable grounds. The plots are separated by distance of hundred metres to several kilometres, which do not constitute any limit to individual mobility. The forests are relatively young and do not offer many alternative breeding cavities, so the majority of the local population breeds in nest boxes. This population has been extensively studied since 1980. All breeding attempts are monitored every year. All nestlings are individually marked, and adults are recaptured while breeding. This allows following individual though their lifetime and measuring their reproductive output. The population is characterized by relatively high, local, recruit production. Up to 12% of locally born offspring recruit to the breeding population, and adult survival is relatively constant across age classes (at $\sim$ 50%). Conse-

quently, this population is particularly suitable for studies of lifetime reproduction. Females were caught at the nest while brooding their young, aged according to previous records (birds were ringed as nestlings or yearlings) and measured for tarsus length and body weight. *c.* 80 μL of blood was drawn from the wing vein to a heparinized capillary. Blood samples were stored in 96% ethanol prior to DNA extraction. Lifespan was measured as a maximum age when the individual was last seen. LRS was measured as the total number of offspring recruiting into the breeding population (i.e. across all years in which each individual bred; see Gustafsson 1989 for details). Using pedigree data and simulations of the possible bias caused by dispersal, LRS was shown to be a valid proxy of a long-term genetic fitness (Brommer *et al.* 2004).

Screening for blood parasites

The prevalence of malaria infection was estimated by PCR assay as described by Waldenström and co-workers (Waldenström *et al.* 2004). The first round of nested PCR was performed in 25-μL volumes. Negative control (deionized water) and positive (*Haemoproteus pallidus* DNA) control samples were always used. Three microlitres of PCR product was visualized on 2% agarose gels stained with ethidium bromide, and positive samples were subsequently prepared for sequencing using FastAP™ (Fermentas). Purified DNA samples were sent to Macrogen Inc., Seoul, Korea, for sequencing. The resulting sequences were aligned and compared with the MalAvi database (Bensch *et al.* 2009) using BioEdit© software.

MHC genotyping

MHC IIB in flycatchers contains both transcribed, apparently functional genes, and nontranscribed, presumably nonfunctional genes. Nonfunctional sequences, which form two clusters on a phylogenetic tree (I and IIA), exhibit indels causing frameshifts (Zagalska-Neubauer *et al.* 2010). To amplify fragment of exon2 of MHC IIB from apparently functional sequences, we used the primers developed by Zagalska-Neubauer *et al.* (2010), FicF1938G and FicR1938, which amplify all known functional alleles, as well as a subset of pseudogene alleles (group II.A of Zagalska-Neubauer *et al.* 2010; exclusion of these pseudogenes was not possible because of the high sequence similarity to some functional alleles; therefore, we filtered out these sequences during subsequent steps of the analysis). Not only do our primers capture the known variation in the study population, but they also show a perfect match to all known sequences of pied flycatchers *Ficedula hypoleuca* (Canal *et al.* 2010). We are therefore confident that the sequences we obtained provide full information on individual-level multilocus genotypes.

For genotyping, we used 454 technology, and based on earlier calculations (Zagalska-Neubauer *et al.* 2010), we aimed at an average coverage of *c.* 1000 reads per amplicon (we used minimum 500 reads/individual for genotyping).

A 197-bp fragment (excluding primers) of class II exon 2 was amplified from 249 samples using fusion primers containing sequences of the primers FicF1938G and FicR1938. Thirty individuals were amplified in duplicates and two in triplicates. The forward fusion primer 5′-GCCTCCCTCGCGCCATCAGNNNNNNGAGTGTCHYTTCVTTAACGGCACG-3′ was composed of the 454 FLX amplicon A primer. A 6-bp tag (indicated with Ns) was used to distinguish individuals. (FicF1938G sequence underlined). The reverse fusion primer consisted of the 454 FLX amplicon B primer and the FicR1938 sequence (underlined) 5′-GCCTTGCCAGCCCGCTCAGCTCTGCGCTCCACGVBGAACGGG-3′. Polymerase chain reaction was performed in 20 μL and contained *c.* 100 ng of genomic DNA, 2 μL of 10× PCR buffer with $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 1 μM of each primer, 0.2 mM of each dNTP and 1 U of *Taq* polymerase (Fermentas). The PCR scheme was the following: 94 °C/3 min, 33× (94 °C/30 s, 58 °C/30 s, 72 °C/30 s) and 72 °C/3 min. The concentration of the PCR product was visually assessed after being run on agarose gels, and the PCR products were pooled into approximately equimolar quantities. The resulting pools were purified using the MinElute PCR Purification Kit (QIAGEN). Purified pools were then sequenced as a part of a single 454 FLX Titanium run according to the 454 Amplicon Sequencing protocols provided by the manufacturer (Roche) at the Functional Genomics Center, Uni/ETH Zurich. As only 35 tagged primers were used, the picotitre plate was divided into eight sections. Reads containing the complete forward primer, complete tag and at least 10 internal bases of the reverse primer were extracted from multifasta files and sorted according to tags; fasta files with alignments of variants present in individual amplicons were generated with the JMHC software (Stuglik *et al.* 2011) available from http://code.google.com/p/jmhc/. Throughout the study, the term 'coverage' refers to the total number of sequencing reads obtained for an amplicon.

Errors occurring during the 454 sequencing include substitutions and small indels (Babik *et al.* 2009; Babik 2010; Galan *et al.* 2010). The frequency of errors resulting in base substitutions was low, and these were expected to occur randomly across the sequence. Therefore, the probability of multiple, identical substitution errors was low (Galan *et al.* 2010). Single base indels

occurring in homopolymer tracts were relatively common in the 454 reads and were nonrandomly distributed along the sequence. However, such variants were excluded from further analyses because functional MHC alleles should not contain frameshift mutations in the coding parts.

Another type of artefact, which will occur with any available MHC genotyping methods, is PCR recombination (chimeras). Chimeras are difficult to analyse because they may be produced repeatedly during PCR by recombination among pairs of true alleles (Longeri *et al.* 2002; Lenz & Becker 2008; Galan *et al.* 2010). Distinct recombination events may produce identical chimeras. Some true alleles may have sequences identical to chimeras because of their evolutionary origin through an in vivo recombination between alleles already present in the population. However, a critical feature distinguishing true recombinants from PCR-derived chimeras is that the latter should always co-occur with both parental sequences in the same amplicon, whereas true recombinants may or may not co-occur with one or both parental sequences. Using this rationale and assuming that artefacts should generally be less abundant than true alleles, we applied the following procedure to distinguish true alleles (197 bp) from PCR artefacts, which was earlier successfully applied to characterize variation in functional MHC IIB genes in collared flycatchers (Zagalska-Neubauer *et al.* 2010).

At least three reads of a variant were required for allele calling, as it is extremely unlikely that three identical sequences occur by chance owing to sequencing errors (Galan *et al.* 2010). However, PCR chimeras may be still present in more copies. Therefore, we started by setting thresholds of sequence variants' frequencies (per amplicon), distinguishing (common) true variants from (rare) artefacts. To this aim, we calculated the maximum per amplicon frequency (MPAF) for each sequence variant and used these values to sort the variants from all amplicons. The least abundant variants were then examined starting from the arbitrarily chosen MPAF value of 0.5%. We selected three (if available) amplicons in which a given variant occurred. We selected amplicons in which the given variant was most abundant (including the one on which the MPAF value was based) and checked them to see whether the variant could be explained as a chimera of more abundant variants within the same amplicon or whether it differed by only 1-bp substitution from one or more common alleles (to account for sequencing errors). On this basis, variants were classified as either an artefact or a true allele.

Working from the bottom of the list, all 127 sequence variants with MPAF 0.5–2.5% were examined. Within the 0.5–1% interval, 90.8% (69 of 76) of the variants were classified as an artefact. Based on this, we also considered all other variants below the 0.5% threshold to be artefacts. Among variants with an MPAF of 1–1.5%, the percentage of artefacts was 53.3 (8 of 15). However, none of the 36 variants with MPAF of 1.5–2.5% could have been classified as an artefact, indicating that all sequences appearing above this threshold can safely be assumed to be true alleles. This was confirmed by a random sampling of 25 variants with MPAF >2.5%, for which none was classified as an artefact. The highest MPAF for a variant classified as artefact was 1.4%. Based on the above thresholds, genotyping was performed for all individual amplicons with a total coverage of at least 500. All variants that occurred with per-amplicon frequency below 0.5% were considered artefacts, whereas those above 2.5% threshold were considered true alleles. The variants in the 'grey zone' (0.5–1.4% per amplicon frequency) were compared to other variants within the same amplicon and considered a true allele only if they could not be chimeras between more common alleles.

There were two very similar alleles: *Fial-DNB*016* and *Fial-DNB*038*, which could not be reliably genotyped as discrepancies concerning their presence/absence occurred in 1/3 of the replicated individuals. These two alleles were divergent from all of the other alleles (min. sequence divergence 25.9%), and it remains unclear whether they are expressed and functional. These alleles were excluded from the analyses.

Twenty-nine true alleles (of the total of 193 alleles) were each present in only a single amplicon. The two-PCR criterion is the standard for MHC studies (i.e. to be confirmed, an allele must be obtained from two independent PCRs, to guard against PCR artefacts Babik 2010). However, we decided to also include alleles obtained from only one amplicon because the stringent procedure we applied to distinguish true alleles from artefacts accounted for the known sources of artefacts, and the problem of mosaic sequences created by bacterial repair of heteroduplexes during traditional cloning (Longeri *et al.* 2002) did not apply. On the other hand, applying the two-PCR criterion would underestimate individual diversity and bias our analyses; therefore, we used sequences that occurred in only one individual in genotyping. Sequences of these alleles are given in Data S1 and S2. In any case, these rare alleles could not appreciably affect our results.

Measures of individual MHC diversity

Before analyses, sequences were translated into amino acids, so that nucleotide sequences differing as a result of synonymous substitutions were considered to be the same allele. The number of unique amino acid sequences per individual constituted our first measure

of individual MHC diversity. The second measure was functional diversity, because functional differences between alleles are mostly defined by properties of amino acids involved in interactions with antigens (Doytchinova & Flower 2005; Schwensow *et al.* 2007). To calculate functional diversity, amino acids involved in binding antigens were scored for five principal-property descriptors, based on 26 physicochemical variables that summarize several key properties of amino acids (such as lipophilicity, steric and electronic properties Sandberg *et al.* 1998). As a rule, sites involved in antigen binding (ABS) show signatures of positive selection in terms of elevated dN/dS ratio (Garrigan & Hedrick 2003; Piertney & Oliver 2006). Rather than relying on the crystallographic models of functionally important amino acids based on human MHC, which are unlikely to be an exact match to those of birds, we followed Schwensow *et al.* (2007) in using amino acids in codon positions that were found to be under historical positive selection in the collared flycatcher. About half of the positions we found to be under positive selection in flycatchers overlapped with human ABS (Fig S1, Supporting information). Each amino acid was characterized by Z-descriptors, and various methods of clustering (hierarchical clustering; k-means clustering; multidimensional scaling) were applied to define functional units called 'supertypes' (Doytchinova & Flower 2005; Schwensow *et al.* 2007). However, none of the methods resulted in alleles being grouped in distinct clusters, precluding the nonarbitrary definition of supertypes. Instead, we calculated indices of within-individual functional MHC diversity based on the sums of pairwise Euclidean distances between z-descriptors of all alleles found in an individual.

Statistical analyses

The impact of historical selection on the MHC sequences was assessed by fitting three models of codon evolution available in PAML (Yang 2007): (i) M0: one ω (dN/dS ratio), (ii) M7: nearly neutral ($\omega \leq 1$) with the beta distribution approximating ω variation and (iii) M8: positive selection (a proportion of sites evolving with $\omega > 1$) with the beta distribution approximating ω variation. The best-fitting models were chosen on the basis of the Akaike information criterion (AIC: Posada & Buckley 2004; Sullivan & Joyce 2005). Positively selected codons were identified through the Bayes empirical Bayes procedure (Zhang *et al.* 2005).

The effect of individual MHC diversity on the presence of parasites and LRS, as well as the effect of infection on LRS, was analysed with generalized linear models, with binomial and quasi-Poisson error distributions, respectively. The effects of MHC diversity and parasites on lifespan were analysed with Cox proportional hazards model. The analyses were carried out using Statistica 8.0. Condition index (a residual from the regression of body mass on tarsus length), female age and the size of her clutch (i.e. number of eggs) in the breeding season when blood sample was collected were included as covariates; these factors are likely to influence the parasite load, the lifespan and the LRS. Because the optimality hypothesis predicts a nonlinear MHC diversity–fitness association, we also fitted the quadratic effect of MHC diversity.

We also analysed the effects of possessing specific MHC alleles on LRS and on the probability of carrying the most common blood parasite we found: the hPHSIB1 strain (*Haemoproteus majoris*, Table S1, Supporting information). The justification for separate analyses of parasite strains is that MHC–parasite associations are expected to be very specific, and thus, joint analyses of all strains are likely to obscure these specific effects. We were only able to analyse the hhPHSIB1 strain because other strains were too rare for a meaningful analysis. We have included all alleles occurring in at least 5% of individuals. As in previous analyses, condition, age and clutch size were used as covariates. Each of the 61 alleles was fitted separately; to protect against false discovery, we calculated *Q*-values (*P*-values corrected for false discovery rate) using QVALUE software (Storey 2002; available from http://genomics.princeton.edu/storeylab/qvalue/).

Results

We obtained 347 196 sequencing reads that can be assigned to amplicons. About 66% of the reads were from clades containing functional alleles, and most other reads were from the group II.A pseudogenes (Zagalska-Neubauer *et al.* 2010). Mean (±SD) coverage was 1244.4 (±474.3) reads ranging from 108 to 2666 reads per amplicon. Using the criteria described in the Methods section, we identified 193 functional alleles in the entire data set. Distribution of allele frequencies for these individuals is presented in Fig. S2 (Supporting information). Among individuals amplified and sequenced in duplicates or triplicates, 27 of 32 had attained coverage of 500 in all replicates. These individuals were used for assessing the genotyping errors. There were no discrepancies among replicates.

The number of alleles (based on both nucleotide and amino acid sequences) per individual ranged from 3 to 23 (average 11.9 ± 3.2; distribution is shown in Fig. S3, Supporting Information). The number of reads in an amplicon did not predict the number of MHC alleles detected ($R^2 = 2 \times 10^{-6}$, $P = 0.98$), indicating we achieved sufficient coverage for reliable genotyping.

The model of codon evolution allowing for positive selection fitted the data better than models without positive selection (Table 1). The Bayes empirical Bayes procedure identified 13 codons under positive selection (Fig. S1, Supporting information). The location of positively selected sites only partly agreed with the location of the ABS as identified by Brown *et al.* (1993), Reche & Reinherz (2003) or Tong *et al.* (2006) based on crystallographic models of human MHC II proteins. It is worth emphasizing, however, that the location of the ABS is only partially concordant among these studies (Fig. S1, Supporting information).

We found 10 strains of blood parasites in our samples, that is, hCOLL2, hCOLL3, hPHSIB1, pACCTAC01, pCOLL7, pGRW09, pGRW10, pPADOM12, pRFF1 and pRTSR1. Their frequencies can be found in Table S1 (Supporting information). In three of our 212 samples (0.014), the sequence data indicated mixed infections (coinfecting strains were not identified).

In total, data for malaria infection and LRS were available for 192 genotyped individuals. The number of amino acid sequences did not predict infection with bird malaria (all strains included; log likelihood $\chi^2 = 2.050$, $P = 0.152$; quadratic effect: log likelihood $\chi^2 = 1.109$, $P = 0.292$). However, there was a significant linear negative association between functional MHC diversity and probability of infection (Table 2, Fig. 1). Infected individuals had lower functional diversity (mean = 1033.5) than uninfected individuals (mean = 1214.9).

The probability of being infected by hPHSIB1, the dominant blood parasite strain in this population, decreased with age (log likelihood $\chi^2 = 4.287$, odds ratio = 0.73, $P = 0.038$; mean age infected = 2.27, SE = 0.17, $n = 52$, mean age uninfected = 2.68, SE = 0.09, $n = 159$). Furthermore, increased susceptibility to infection was associated with the *Fial-DNB*63* allele (log likelihood $\chi^2 = 9.194$, $P = 0.002$), but this association was not significant after false discovery correction (Q value = 0.132). Several other alleles (**7*, **9*, **33*, **42* and **44*) reached P values <0.05 (associated Q-values > 0.22). Infection with bird malaria tended to be associated with decrease in LRS, but the effect was not statistically significant (log likelihood $\chi^2 = 2.459$, $P = 0.116$); separate analysis of the effect of being infected with the hhPHSIB1 strain yielded a similar result (log likelihood $\chi^2 = 2.746$, $P = 0.097$). Neither infection with bird malaria ($\chi^2 = 0.478$, $P = 0.489$) nor with the hhPHSIB1 strain ($\chi^2 = 0.399$, $P = 0.527$) significantly affected female lifespan.

The number of amino acid sequences did not significantly predict the LRS (log likelihood $\chi^2 = 0.872$, $P = 0.350$; quadratic effect: log likelihood $\chi^2 = 1.095$, $P = 0.294$). Likewise, there was no significant association between functional MHC diversity and LRS (Table 2). Neither the number of amino acid sequences ($\chi^2 = 0.060$, $P = 0.800$) nor functional MHC diversity

Table 1 Evaluation of fit for different models of codon evolution and estimated parameter values

Model	lnL	δAIC	Parameters
M0, one ω	−4952.6	943.8	ω = 0.602
M7, nearly neutral with beta	−4518.9	78.4	
M8, positive selection with beta ($\omega_0 \leq 1$, $\omega_1 > 1$)	−4477.7	Best	$p_0 = 0.761$, $p_1 = 0.239$, $\omega_1 = 2.357$

ω − dN/dS; nearly neutral with beta—for all sites ω ≤ 1 and the beta distribution approximates ω variation; positive selection—a proportion of sites evolves with ω > 1; p_0, proportion of sites with ω ≤ 1, p_1, proportion of positively selected sites (ω > 1), ω_1, estimated value of ω for sites under positive selection; δAIC, the difference between the value of the Akaike information criterion (AIC) of a given model and the best model.

Table 2 A generalized linear model testing association among MHC II functional diversity within individuals (see Methods) and infection with blood parasites and lifetime reproductive success (LRS)

	Infection		LRS	
	χ^2	P	χ^2	P
MHC diversity	4.840	0.028	1.670	0.196
MHC diversity^2	2.847	0.091	2.798	0.094
Clutch size	0.021	0.884	2.481	0.115
Age	0.262	0.608	28.70	<0.001
Condition	0.247	0.616	2.677	0.101

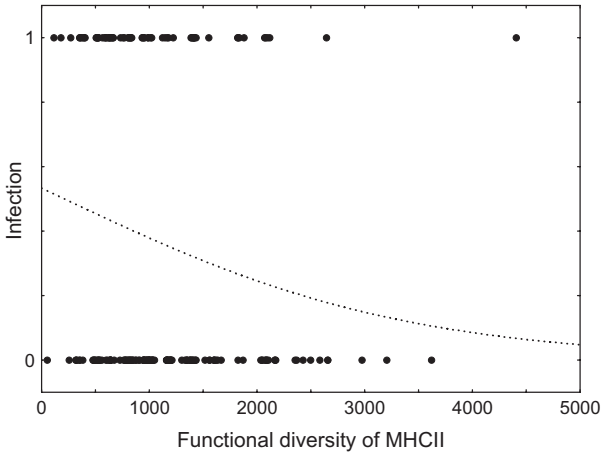

Fig. 1. Relationship between functional diversity of MHC alleles and the probability of infection with blood parasites. 1, infected; 0, uninfected. Broken line was fitted with logistic regression.

($\chi^2 = 0.307$, $P = 0.579$) affected female lifespan. Models testing effects of individual alleles indicated that *FialDNB*022* is associated with LRS ($\chi^2 = 5.23$; $P = 0.022$), but this effect was not significant after correction for false discovery rates (*Qvalue* = 0.756).

Discussion

We investigated whether individual MHC diversity impacts malarial infection and LRS. Such associations are thought to shape variation in MHC copy number, which is considerable both within (e.g. Wegner *et al.* 2003a; Bonneaud *et al.* 2004; Meyer-Lucht & Sommer 2009; Kloch *et al.* 2010) and between (Kelley *et al.* 2005) species. The present study confirmed the extreme between-individual variation in the number of functional MHC alleles in the collared flycatcher reported earlier by Zagalska-Neubauer *et al.* (2010). The maximum number of alleles in the present study was 23, suggesting at least 12 expressed loci.

Such extensive variation, resulting either from differences in the number of loci or from allele sharing between loci, allowed us to test the optimality hypothesis. The hypothesis assumes that while producing a highly diverse repertoire of MHC proteins allows for binding of a larger antigen spectrum, it is also associated with costs. These costs may be due to negative selection limiting the lymphocyte repertoire in highly MHC-diverse individuals (Nowak *et al.* 1992; Woelfing *et al.* 2009). However, we detected a significant negative relationship between the probability of malarial infection and functional MHC diversity. Thus, our results is not consistent with the idea that this relationship should be nonlinear, as is predicted by the optimality hypothesis. In contrast, nonlinear relationships have been reported in sticklebacks (Wegner *et al.* 2003b), water pythons (Madsen & Ujvari 2006) and bank voles (Kloch *et al.* 2010). Research on sticklebacks and bank voles looked at the effects of several pathogen species coinfecting individuals, whereas in flycatchers we only looked at bird malaria. The prediction of the optimality hypothesis—that individuals with extremely low or extremely high within-individual MHC diversity will have more difficulty in resisting parasites—should hold for any parasite but could be easier to detect when a greater number of parasite species is sampled simultaneously. Nevertheless, the significantly negative association we report is clearly at odds with the predictions of the optimality hypothesis.

While increased MHC diversity was associated with decreased prevalence of bird malaria, the association was not reflected in longer lifespan or higher reproductive success of the most MHC-diverse individuals, indicating that malaria infection, as measured in adult females, is not a strong determinant of LRS. Indeed, we have not detected a significant effect of malaria on lifespan or LRS. The avian blood parasites were for a long time regarded as benign to their hosts, as correlative studies (Fallis & Desser 1977) reported little evidence of detrimental effects of infection. However, experimental studies have shown that even chronic infections can have a major impact on the host fitness. For instance, La Puente *et al.* (2010) showed in their medication experiment that only individuals that had their parasite load reduced or completely cleared (in this study, only females) exhibited increased local survival to the next breeding season. Moreover, some studies suggest that malaria infections may decrease parental effort and therefore impair fitness of the offspring. In a classical study by Merino *et al.* (2000), the blue tit females that were medicated against blood parasites with primaquine experienced higher fledgling success compared to control females. In accord with this study, Knowles *et al.* (2010) found that females with reduced or cleared malaria infections showed higher hatching success, provisioning rate during feeding period and fledgling success. Altogether, these studies support the claim that malaria parasites may have a significant impact on LRS in their avian hosts. However, such effects may not be easy to detect in correlational studies.

We have found a significant decrease in the prevalence of hPHSIB infection with age. This can be explained in two ways. First, young individuals may be more susceptible to infection, or infection may be contracted early and then combated with age. Secondly and probably more likely, infection with blood parasites may make it more difficult for birds to survive, such that infected young birds are less likely to enter higher age classes. Indeed, avian malaria can be especially acute at the first exposure, but once controlled, it may have low-to-moderate fitness effects (Atkinson & van Riper 1991; Cellier-Holzem *et al.* 2010).

We have not found any evidence to support the effect of MHC diversity on LRS. Other studies have found some evidence for fitness consequences of MHC copy number variation. Bonneaud *et al.* (2004) reported that the nestlings of female house sparrows with an intermediate number of alleles survived better than the offspring of females with extreme values. Intermediate copy number females also laid larger first clutches, although their second clutches were smaller. Therefore, the net consequences of MHC diversity on fitness in house sparrows are not clear. In contrast, a long-term study of the Seychelles warbler revealed a positive association between the number of alleles/individual and juvenile survival (but not adult survival, Brouwer *et al.* 2010). Results published to date are thus inconsistent and concern only some fitness components, such that

they reveal only a partial picture of fitness consequences of MHC diversity.

In the only study to date that has investigated the effect of MHC diversity on LRS, sticklebacks with intermediate diversity achieved the highest fitness (Kalbe *et al.* 2009). In contrast, our results did not provide evidence for selection favouring an optimal MHC diversity. The lack of the significant association of lifespan or LRS with MHC diversity may be due to the fact that estimating fitness in the wild is challenging and may not be very accurate. Measure of LRS assumes that whole lifespan reproduction is recorded, nonrecruiting offspring do not survive, and there is no bias in dispersal. Despite these caveats, LRS proved to reflect well the genetic fitness calculated with simulations taking dispersal into account (Brommer *et al.* 2004). We thus conclude that if selection plays a role in shaping individual MHC diversity in flycatchers, it is probably very weak. Indeed, it may be that the high MHC copy number variation, as observed in collared flycatchers, is possible only under weak selection against high diversity: if optimizing selection was strong, we should probably expect a narrower variation.

We also investigated whether the most common MHC alleles occurring in our study population are associated with malarial infections and with the LRS of female flycatchers. Associations between avian malarial strains and MHC types were earlier reported in reed warblers (Westerdahl *et al.* 2005) and house sparrows (Bonneaud *et al.* 2006; Loiseau *et al.* 2008, 2011).These studies only investigated associations of bird malaria with MHC class I genes, but malaria has been shown to be associated with MHC class II alleles in humans (e.g. Oliveira-Ferreira *et al.* 2004), and antibodies were found to be produced (e.g. against epitopes present on free merozoites; Johnson *et al.* 2004). Our analyses indicated several candidate alleles that could be associated with malaria infection (especially the *Fial-DNB*63* allele), but with the present sample we could not detect any significant association after correction for false discovery rate. Likewise, allele *Fial-DNB*22* tended to be associated with lower reproductive success, but this effect was not significant after correcting for false discovery rate. Low power of statistical tests, even with reasonable sample sizes, is an inherent problem in a system with many predictor variables (here: MHC alleles). A solution proposed by Kaslow *et al.* (1996) is to treat candidate alleles found in a first survey as a priori predictors in subsequent analysis. Future work on flycatchers may trace changes in frequencies of candidate fitness-associated alleles found in this study, in the following generations.

Overall, our results suggest that high, functional MHC diversity might give individuals an advantage in some infections. However, the diversity does not seem to have major fitness consequences in flycatchers.

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Data accessibility

DNA sequences (FASTA format) and a spreadsheet containing the data (Excel) are deposited in DRYAD (doi:10.5061/dryad.c80675fg); additionally, the sequences are listed in Data S1 and S2.

Supporting information

Additional supporting information may be found in the online version of this article.

Data S1 A list of sequences (in FASTA format), not including sequences which appeared in single individuals only (these are given in a separate list).

Data S2 A list of sequences which appeared in single individuals (in FASTA format).

Table S1 Diversity of avian malaria lineages in collared flycatchers from Gotland. Prevalence of lineages can be obtain by

dividing the number of positives by the total screened (212 individuals).

Fig. S1 A sequence logo showing the relative frequencies of various amino acids in particular positions of the examined fragment of the MHC class IIB 2nd exon.

Fig. S2 Frequency distribution of MHC II B alleles in Gotland population of collared flycatchers.

Fig. S3 Distribution of the number of alleles per individual flycatcher.

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