

## ORIGINAL ARTICLE OPEN ACCESS

# MHC Class II Supertypes Affect Survival and Lifetime Reproductive Success in a Migratory Songbird

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## ABSTRACT

The major histocompatibility complex (MHC) plays a critical role in the immune response against pathogens. Its high polymorphism is thought to be mainly the consequence of host–pathogen co–evolution, but elucidating the mechanism(s) driving MHC evolution remains challenging for natural populations. We investigated the diversity of MHC class II genes in a wild population of pied flycatchers *Ficedula hypoleuca* and tested its associations with two key components of individual fitness: lifetime reproductive success and survival. Among 180 breeding adults in our study population, we found 182 unique MHC class II exon 2 alleles. The alleles showed a strong signal of positive selection and grouped into nine functional supertypes based on physicochemical properties at the inferred antigen-binding sites. Three supertypes were found in >98% of the sampled individuals, indicating that they are nearly fixed in the population. We found no rare supertypes in the population, as all supertypes were present in >70% of individuals. Three supertypes were related to different components of individual fitness: two were associated with lower offspring production over time, while the third was positively associated with survival. Overall, the substantial allelic and functional diversity and the relationship between specific supertypes and fitness are in accordance with the notion that balancing selection maintains MHC class II diversity in the study population, possibly with fluctuating selection as the underlying mechanism. The absence of rare supertypes in the population suggests that the balancing selection is not driven by rare-allele advantage.

David Canal and Jacob Roved shared first authorship.

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## 1 | Introduction

Understanding the genetic basis of fitness differences is a central issue in evolutionary biology (Ellegren and Sheldon 2008). The ability of an organism to protect itself from pathogens and parasites has a major impact on fitness, either directly (e.g. by increased survival among individuals with more optimal immune responses) or indirectly (e.g. through trade-offs against other life-history components such as growth or reproduction; Kalbe et al. 2009; Sepil, Lachish, and Sheldon 2012; Thoß et al. 2011), and thus influences selection on immune response genes. The major histocompatibility complex (MHC) is a group of genes widely found in vertebrates that play an essential role in the adaptive immune response (Klein and Sato 2000). MHC genes code for molecules that present pathogen-derived antigens to T lymphocytes and initiate immune responses against pathogens (Trowsdale 1993). Although its involvement in resistance or susceptibility to different pathogens has been widely studied, there is still controversy about the exact mechanism underlying MHC evolution (Lighten et al. 2017) and how MHC variation influences the fitness of individuals in wild populations (Ejsmond and Radwan 2015; Kaufman 2018; Radwan et al. 2020).

MHC is the most polymorphic group of genes known in vertebrates. Balancing selection driven by host-pathogen co-evolution is thought to be the main reason for the extreme MHC polymorphism, although other mechanisms may be involved (Ejsmond and Radwan 2015; Kamiya et al. 2014; Kaufman 2018; Lenz 2011; Lighten et al. 2017; Roved et al. 2018). Several non-mutually exclusive mechanisms of pathogen-mediated selection have been proposed for maintaining MHC diversity. First, heterozygous individuals may have an advantage in expressing MHC molecules capable of targeting a wide range of pathogens (Doherty and Zinkernagel 1975; Oliver, Telfer, and Piertney 2009). This is the basis of the maximum diversity hypothesis, which posits that fitness increases with individual MHC diversity. However, under the optimal diversity hypothesis, an intermediate, optimal MHC diversity, rather than a maximum diversity, is thought to confer an advantage because a too diverse set of MHC alleles may limit the antigen-recognition repertoire of T-cells as a result of negative selection during thymopoiesis (Migalska, Sebastian, and Radwan 2019; Nowak, Tarczy-Hornoch, and Austyn 1992; Woelfing et al. 2009). Second, MHC diversity may be maintained by directional selection on specific MHC alleles that either provide resistance or increase susceptibility to specific pathogens (Aguilar et al. 2016; Westerdahl et al. 2012). Under this scenario, allele-specific selection may fluctuate in time and space depending on variation in pathogen pressures irrespective of allele frequency (fluctuating selection hypothesis). Nevertheless, selection may also be associated with the frequency of alleles in the population, as pathogens are more likely to evolve to evade detection by the most common MHC alleles, which may confer an advantage to individuals carrying rare alleles (rare-allele advantage hypothesis) (Bodmer 1972; Borghans, Beltman, and De Boer 2004). Under this latter hypothesis, the frequency of a protective, rare allele in the host population would increase until pathogens adapt to it, and the co-evolutionary process would favour the emergence and spread of other MHC alleles (Ejsmond and Radwan 2015).

Many studies have investigated the effects of MHC at the allele level, based on either allelic diversity or specific alleles, on different proxies of fitness. However, these measures of genetic make-up may not be good indicators of the ability of individuals to cope with multiple pathogens. For example, individuals with high allelic diversity may possess similar alleles, while those with low allelic diversity may have sufficiently divergent alleles to respond to different potential pathogens (Pierini and Lenz 2018; Wakeland et al. 1990). Therefore, functional divergence between MHC alleles should be taken into account (Lighten et al. 2017). An efficient approach to characterising this is the classification of MHC alleles into supertypes based on the physicochemical properties of the amino acid positions involved in antigen binding (Lighten et al. 2017; Reche and Reinherz 2007; Roved 2022; Sette and Sidney 1998). Possessing specific MHC supertypes has been shown to confer an immunological advantage in several studies (Buczek et al. 2016; Kubinak et al. 2012; Sepil, Lachish, and Sheldon 2012; Sepil et al. 2013) and, as proposed by Lighten et al. (2017), considering these functional aspects may be crucial to elucidate the evolutionary mechanisms of polymorphic genes involved in host–parasite co-evolution.

MHC composition may have differential effects on fitness at different life stages (e.g. early life, breeding, migration), and its effects may be sensitive to the environmental conditions (favourable vs. adverse conditions) experienced by individuals (Guillaume et al. 2023; Lau et al. 2020; Minias, Whittingham, and Dunn 2017; O'Connor et al. 2019; Roved, Westerdahl, and Hasselquist 2017; Zuk and Stoehr 2002). Thus, investigating the influence of MHC on various fitness proxies covering the entire life cycle of individuals is essential for reliably detecting MHC-fitness effects and understanding the mechanisms driving MHC evolution (Näpflin et al. 2019; O'Connor et al. 2019). However, following individuals from birth to death is challenging in natural populations, and few studies have been able to test MHC variation in relation to lifetime reproductive success and lifespan in the wild (Huang et al. 2022; Kalbe et al. 2009; Radwan et al. 2012; Roved et al. 2018; Sepil, Lachish, and Sheldon 2012).

Here, we use a 10-year individual-based longitudinal dataset from a population of pied flycatchers (*Ficedula hypoleuca*) to investigate whether quantitative or qualitative aspects of variation in the MHC class II affect individual fitness, measured as lifetime reproductive success (number of fledglings produced over an individual's lifetime; LRS) and survival. Based on the complete characterisation of the exon 2 of MHC class IIB, previous work in this population has shown considerable allelic diversity and between-individual variation in the number of functional MHC class II genes (Canal et al. 2010). Pied flycatchers, including our study population, show high breeding-site fidelity (Lundberg and Alatalo 1992; Canal, Dávila, and Potti 2011; Camacho et al. 2019; Potti et al. 2021), which allows monitoring of individuals throughout their lives and makes the species an excellent system to study MHC evolution. Specifically, we aimed to test the predictions of three hypotheses relating to MHC and fitness: (i) fitness increases as MHC-II diversity increases (maximum diversity hypothesis), (ii) the highest fitness is associated with intermediate MHC-II diversity (optimal diversity hypothesis) and (iii) variation in fitness is associated with specific MHC-II supertypes, potentially indicating fluctuating selection or rare-allele advantage.

## 2 | Materials and Methods

### 2.1 | Study System

Data were collected from a long-term study population of pied flycatchers breeding in nest boxes in La Hiruela, Central Spain (ca. 41°4'42" N, 3°25'55" W, 1200–1300 m a.s.l.). The pied flycatcher is a long-distance migratory songbird that exhibits sexual dimorphism in plumage colour during the breeding season. The species has a predominantly monogamous mating system, although some males (<10%; Canal et al. 2020) may occupy a second nest cavity, attract a second female and become socially bigamous. The study area consists of two plots, a natural oak forest (*Quercus pyrenaica*) and a conifer plantation dominated by *Pinus sylvestris*, separated by 1.1 km. There are 237 nest boxes (156 in the oak forest and 81 in the pine plantation) distributed at approximately 20-m intervals in both plots (Camacho et al. 2019).

Blood samples for MHC genotyping were obtained from 181 adults during two consecutive breeding seasons in 2005 and 2006. The capture (breeding) histories of these individuals spanned from 2000 to 2011, excluding 2003 due to limited sampling effort (Potti et al. 2021). To ensure comparability, we excluded data from individuals involved in experiments (e.g. cross-fostering) conducted during the long-term study, resulting in 112 breeding adults (57 females and 55 males) available for this study. All breeding adults were monogamous, and their reproductive history was known, which allowed reliable estimates of survival and LRS to be obtained from this nest-box study.

### 2.2 | Fieldwork and General Procedures

Field protocols have been described in detail elsewhere (Camacho, Canal, and Potti 2013; Canal, Dávila, and Potti 2011). Briefly, during the breeding season, which lasts from around the third week of April (when first males arrive from migration) to the first 2 weeks of July, all nests were regularly checked to determine laying date, clutch size (typically 5–6 eggs), hatching date and number of fledglings. Fledglings were ringed, measured and weighed at 13 days of age. Breeding adults were captured with a nest-box trap while feeding eight-day-old nestlings, measured for standard morphological traits, and individually marked with a numbered metal ring and a unique combination of colour rings (only males). Due to the strong natal- and breeding-site fidelity of individuals in the study population (Canal, Serrano, and Potti 2014; Potti, Canal, and Serrano 2013; Potti and Montalvo 1991), ca. 53% of breeding adults are birds ringed as nestlings in our nest boxes (Potti and Montalvo 1991). Unringed breeders were aged as first year or older based on plumage traits (Karlsson, Persson, and Walinder 1986; Potti and Montalvo 1991). Blood samples were taken from all individuals by puncturing the brachial vein and stored in absolute ethanol.

### 2.3 | MHC Genotyping

DNA was extracted using the Qiagen blood extraction kit. We used the primers MHCIIIFihy-E2CF and MHCIIIFihy-E2CR, located in the introns-exon 2 junction, to amplify the entire coding region of exon 2 (270 bp) across the multigene family of

functionally relevant MHC class II B genes in pied flycatchers (see Canal et al. 2010 for details).

MHC library preparation and sequencing were performed by LGC Genomics GmbH (Berlin, Germany) on an Illumina MiSeq (Illumina, Inc., CA, USA). Details on the library preparation protocol and sequencing (instrument, chemistry, facility) are given in Data S1.

#### 2.3.1 | Technical Replicates and Repeatability Assessment

Our sequencing dataset included 43 sets of technical replicates which were amplified in independent PCRs and sequenced with individual barcodes. The repeatability of our sequencing data was calculated as 1 minus the mean across all replicate sets of the mean proportion of mismatching sequence variants within each replicate set. Replicate sets were compared using ReplMatch, and repeatability was calculated using GetReplStats from the R package MHCtools v.1.5 (Roved 2022).

#### 2.3.2 | Filtering of the Illumina Sequencing Data

The mean number of reads per sample in the raw Illumina sequencing data was 48,275.54 with SD = 7540.67. Before filtering the Illumina sequencing data, reads with length <150 bases were removed from both forward and reverse read files (while maintaining the coordination of read pairs in forward and reverse files). Custom R scripts and MHCtools v.1.5 were then used to loop filtering over different settings in dada2 v.1.30.0 (Callahan et al. 2016) and carry out replicate matching to find optimal settings, following the genotyping workflow described in Roved (2024). Details on the filtering optimisations are provided in the Data S2. Subsequently, the dataset was inspected manually and sequences with length polymorphisms ( $n=5$ ) and stop codons ( $n=7$ ) were removed and sequences aligned and trimmed to open reading frame. We excluded one outlier sample (EA1114) with only two sequences and 11 reads. After these filtering steps, there was no association between the number of sequences and the number of reads per sample in the dataset (linear regression,  $p=0.571$ , adj.  $R^2=-0.003$ ), indicating that our genotyping results are reliable. Finally, the replicated samples were merged by summing the read numbers for the technical replicates.

#### 2.3.3 | Phylogenetic Models

Phylogenetic models of the MHC-II sequences were run in PhyML (Guindon et al. 2010; Guindon and Gascuel 2003). In total, 12 models were run and AIC-based model selection was performed to identify the optimal phylogeny (details are provided in the Data S3). The phylogenetic tree was visualised using the R package ggtree v. 3.10.1 (Yu et al. 2017).

#### 2.3.4 | Test for Positive Selection

Codeml from the PAML software package (Yang 1997, 2007) was used to test for positive selection on the MHC-II sequences.

The software was set to calculate codon frequencies from the average nucleotide frequencies at the three codon positions and to assume one dN/dS ( $\omega$ ) ratio for all branches in the phylogeny. We ran the nested site models M1 (nearly neutral selection) versus M2 (positive selection), and M7 ( $\omega$  following a beta distribution) versus M8 ( $\omega$  following a beta distribution with an additional category of  $\omega > 1$ ), and compared them using likelihood ratio tests (LRT) with the formula:  $2(\ln L_2 - \ln L_1) \sim X^2$  (2 d.f.), where the degrees of freedom of the chi-square distribution was equal to the difference in number of parameters between the models.

### 2.3.5 | Inference of MHC-II Supertypes

MHC supertypes were inferred following the workflow described in Roved (2024). In brief, the amino acids of the positively selected sites of each sequence were characterised by five physicochemical descriptors: z1 (hydrophobicity), z2 (steric bulk), z3 (polarity), z4 and z5 (electronic effects; Sandberg et al. 1998) using the DistCalc function in MHCtools. The z-descriptor matrices were subjected to bootstrapped clustering analysis using BootKmeans in MHCtools with the settings threshold = 0.01, max\_k = 40, nstart = 20, algorithm = 'Hartigan-Wong'. The number of bootstrap models was 1000 and among those, BootKmeans inferred  $k$  values from 8 to 16. ClusterMatch in MHCtools was used to evaluate the output from BootKmeans. This analysis showed that  $k = 9$  was associated with the lowest proportion (0.029) of sequences assigned to low-ranking clusters. Twelve models that inferred  $k = 9$  shared the lowest residual BIC = 11985.01 and among those, the cluster assignments were consistent between models, that is, the identical sequences were assigned to the nine clusters. These nine clusters constitute the MHC-II supertypes, which were employed in downstream analyses.

## 2.4 | Statistical Analyses

### 2.4.1 | Effect of Neutral Genetic Diversity on Fitness

To disentangle an effect of the diversity at the MHC level on fitness variation from an effect caused by genome-wide diversity, we included an index of neutral diversity (linear and quadratic effect) in models related to LRS and apparent survival (product of true survival and the probability that the individual has not permanently emigrated from the sampling area). In particular, we used homozygosity by loci (HL), based on 15 microsatellites loci (see Canal, Serrano, and Potti 2014 for further details), as a proxy of genome-wide diversity because this metric correlates better than others with genome-wide homozygosity and inbreeding in open populations (Aparicio, Ortego, and Cordero 2006). HL varies between 0, when all loci in the individual are heterozygous, and 1, when all loci are homozygous. A 2-year (2005–2006) study involving all breeding individuals in the population, including those of this study, showed that HL ranges between 0 and 0.56, with a mean of 0.208 ( $\pm 0.102$  SD; Canal, Serrano, and Potti 2014). That study also found that the genetic relatedness of parents at this set of loci negatively affected offspring survival, suggesting that this set of loci is informative on the genome-wide heterozygosity of individuals (see also Forstmeier et al. 2012; Taylor et al. 2010).

### 2.4.2 | Effect of MHC Class II Composition on LRS

We used generalised linear mixed models (GLMM; see fitting details below) to investigate the effect of MHC genotype on LRS, measured as the number of fledglings produced over an individual's lifetime. Initially, we explored the best global model structure by considering all possible combinations of the additive effects of 'sex' and 'habitat' (factor with two levels: conifer plantation and oak forest), along with 'year of birth' (factor with seven levels: 1999–2005) as a random intercept. Model selection was performed using the corrected Akaike information criterion for small samples (AICc; Burnham and Anderson 2002). The analysis indicated that the model with only the random intercept was the best supported and was used as the base model's structure for the rest of the analyses. Before fitting the models, we compared the goodness-of-fit of several candidate models using different error distributions (Conway–Maxwell–Poisson, Poisson and negative binomial) using AICc (Burnham and Anderson 2002). This analysis showed that the best-fitting model (lowest AICc) was the one with a Conway–Maxwell–Poisson distribution for errors (results not shown).

Next, we evaluated the maximum and optimal diversity hypotheses by adding the linear and quadratic effects, respectively, of the total number of supertypes per individual. We evaluated the influence of specific MHC supertypes on LRS, adding the presence/absence of each of the six supertypes described in our study population (see below) as the focal predictor. This tested whether, as expected by the fluctuating selection and rare-supertype advantage hypotheses, the presence of a specific supertype affected LRS. In total, we fitted eight models in addition to the two models with the linear and quadratic effect of HL that we ran to discard the effect caused by genome-wide diversity (Table 1).

Model fits were ranked from most to least supported by comparing their AICc values with that of the null model (including only 'year of birth' as a random intercept) (Burnham, Anderson, and Huyvaert 2011). Before interpreting any model, we systematically performed several diagnostic statistics (e.g. distribution of residuals, multicollinearity) to avoid misleading results due to statistical artefacts. These statistics did not indicate any obvious deviation from the assumptions of linear models. All analyses were performed in R 4.3.3 (R Core Team 2024) using the function *glmmTMB* from the homonym package (Brooks et al. 2017). We used the function *simulateResiduals* (package DHARMA; Hartig 2016) and *VIF* (package *car*) for model diagnostics. The R script and results are available in Data S4.

### 2.4.3 | Effect of MHC Class II Composition on Apparent Survival

#### 2.4.3.1 | Multievent Capture–Recapture Modelling.

To estimate apparent survival ( $\Phi$ ) and recapture probabilities ( $p$ ), we used RMark (Laake 2013) in R to fit capture–mark–recapture Cormack–Jolly–Seber (CJS) models. Prior to the capture–mark–recapture analysis, we found adequate support for the CJS model with time variation in apparent survival and recapture probabilities running a goodness-of-fit test using the *R2ucare* package (Gimenez et al. 2018) in R (see Data S5 and S6). We relied on AICc (Burnham, Anderson,

**TABLE 1** | Generalised linear models used to estimate the effect of the MHC diversity on the lifetime fledgling numbers of pied flycatchers.

Model	Predictor	np	$\beta$	SE	AICc	$\Delta$ AICc
2	<b>Supertype 3</b>	<b>4</b>	<b>-0.34</b>	<b>0.11</b>	<b>646.82</b>	<b>-6.16</b>
5	Supertype 7	4	-0.22	0.1	650.13	-2.85
9	nStypes <sup>2</sup>	5	-0.9	0.49	651.7	-1.28
6	Supertype 8	4	0.15	0.1	652.68	-0.3
7	nStypes	4	-0.07	0.04	652.93	-0.05
11	Null	3	NA	NA	652.98	0
3	Supertype 5	4	0.22	0.16	653.06	0.08
8	HL	4	0.04	0.05	654.26	1.28
4	Supertype 6	4	-0.04	0.1	654.91	1.93
1	Supertype 2	4	0.07	0.16	654.92	1.94
10	HL <sup>2</sup>	5	0.34	0.45	655.91	2.93

Note: Selection models were based on Akaike information criterion corrected for small sample sizes (AICc). The model shown in bold is the most parsimonious model. Abbreviations:  $\beta$ , effect of the focal predictor (quadratic term for models 9 and 10);  $\Delta$ AICc, difference in AICc between the null model and the AICc of each model; np, number of estimable parameters; nStypes and nStypes<sup>2</sup>, linear and quadratic covariate of the number of supertypes, respectively; SE, standard error.

and Huyvaert 2011) to determine the relative support of each model according to the principle of parsimony (i.e. the lower the better). Our model selection procedure included a preliminary phase to control for the effects of potential confounding factors such as the age of first reproduction, sex, cohort and habitat (see Data S5 and S6), followed by a final phase with eight models (Table 2). This method was used to test the *maximum and optimal diversity hypotheses*, using the standardised (zero mean and unit standard deviation) number of supertypes (linear or quadratic term, respectively) of each individual as the predictor, and the *fluctuating selection and rare-supertype advantage hypotheses*, using the presence of each supertype (six models) as the focal predictor. As above, we fitted two models including the linear and quadratic effects of HL to control for the potential effect of neutral diversity on apparent survival variation.

### 3 | Results

#### 3.1 | The Pied Flycatcher MHC Class II Exon 2

We found a total of 182 MHC-II alleles, all of them 282 bases long, after filtering, manual inspection and trimming to open reading frame. The repeatability of the final sequencing dataset was 0.969. The number of alleles per individual was normally distributed with mean = 18.2, min = 9, max = 27 and SD = 2.63. The fact that individuals had up to 27 alleles suggests that haplotypes in the pied flycatcher can harbour at least 14 MHC class II loci.

We ran two sets of nested models in Codeml from the PAML software package (Yang 1997, 2007), which both showed evidence of positive selection on the MHC-II sequences ( $p < 0.0001$ , see Tables in Data S7). The M8 model had the largest likelihood value and, in this model, 13.5% of the codons are estimated to

be under positive selection ( $\omega = 3.16$ ). The positively selected sites ( $n = 13$ ) predicted by Bayes Empirical Bayes (BEB) analysis (Yang, Wong, and Nielsen 2005) are shown in Data S8.

The supertype analysis inferred 9 MHC-II supertypes among the 182 alleles, based on z-descriptors that quantify the physico-chemical properties of amino acids in the 13 positively selected sites. The number of alleles per supertype ranged between 4 and 60, and individuals had, on average, 8 MHC-II supertypes (range: 4–9 supertypes per individual). However, three supertypes (supertypes 1, 4 and 9) were found in almost all genotyped individuals (frequency > 0.98) and thus were discarded from further analyses. The frequency of the remaining supertypes varied between 0.67 and 0.9 (Figure 1a). Three supertypes (2–4) comprised alleles that formed monophyletic clades in the phylogenetic analysis of the full-length DNA sequences, while the remaining supertypes (1 and 5–9) were all characterised by admixture of alleles from different clades (Figure 1b).

#### 3.2 | Effect of MHC Class II Composition on LRS

Contrary to the predictions of the *maximum and optimal diversity hypotheses*, neither linear nor quadratic effects of MHC class II diversity were related to LRS (Table 1; models 7 and 9 vs. null model), nor were neutral diversity indices (models 8 and 10). By contrast, individuals with supertypes 3 and 7 produced fewer fledglings over their lifetime than individuals without these supertypes (Figure 2).

#### 3.3 | Effect of MHC Class II Composition on Apparent Survival

We found no support for the maximum and optimal diversity hypotheses' prediction that the MHC class II diversity (either

**TABLE 2** | Results of capture–recapture analyses modelling the effect of MHC diversity on apparent survival probabilities ( $\Phi$ ) of pied flycatchers.

No.	Model	np	$\beta$	SE	Deviance	AICc	$\Delta$ AICc
7	<b>BaseModel + ST5</b>	7	<b>1.67</b>	<b>0.79</b>	<b>206.36</b>	<b>258.36</b>	<b>−3.85</b>
11	BaseModel	6	NA	NA	212.35	262.21	0
6	BaseModel + ST3	7	−0.58	0.41	210.39	262.38	0.17
9	BaseModel + ST7	7	−0.47	0.35	210.52	262.52	0.31
10	BaseModel + ST8	7	0.37	0.35	211.23	263.22	1.01
5	BaseModel + ST2	7	0.34	0.58	211.99	263.99	1.78
8	BaseModel + ST6	7	0.2	0.34	212.01	264.01	1.8
1	BaseModel + HL	7	0.09	0.15	212.02	264.02	1.81
3	BaseModel + nStypes	7	0.01	0.16	212.35	264.34	2.14
2	BaseModel + HL <sup>2</sup>	8	11.54	205.67	212.02	266.18	3.97
4	BaseModel + nStypes <sup>2</sup>	8	10.77	218.38	212.35	266.5	4.29

Note: The base model corresponds to ‘ $\Phi$  (time)  $p$  (constant)’, where the probability of survival ( $\Phi$ ) depends on the year. Resighting probability ( $p$ ) was modelled as constant. The model shown in bold is the most parsimonious model.

Abbreviations:  $\beta$ , effect of the focal predictor (quadratic term for models 2 and 4);  $\Delta$ AICc, difference in AICc between the base model and the AICc of each model; ‘+’, indicates additive effects; AICc, Akaike information criterion corrected for small sample sizes; HL and HL<sup>2</sup> linear and quadratic covariate of the standardised homozygosity by loci; np, number of parameters; nStypes and nStypes<sup>2</sup>, linear and quadratic covariate of the standardised number of supertypes, respectively; SE, standard error; ST, supertype.

mediated or not by neutral diversity) is related to individual survival (Table 2, models 3 and 4 vs. base model). However, we found support for the effect of a specific supertype, supertype 5, on pied flycatcher survival rates (Table 2, model 7 vs. base model). On average, individuals carrying supertype 5 had a higher probability of survival ( $\beta=1.67$ , SE=0.79) than those without that supertype (Figure 3). This model was the best supported among candidate models (Table 2), and the 95% CI of the coefficient estimates did not include zero.

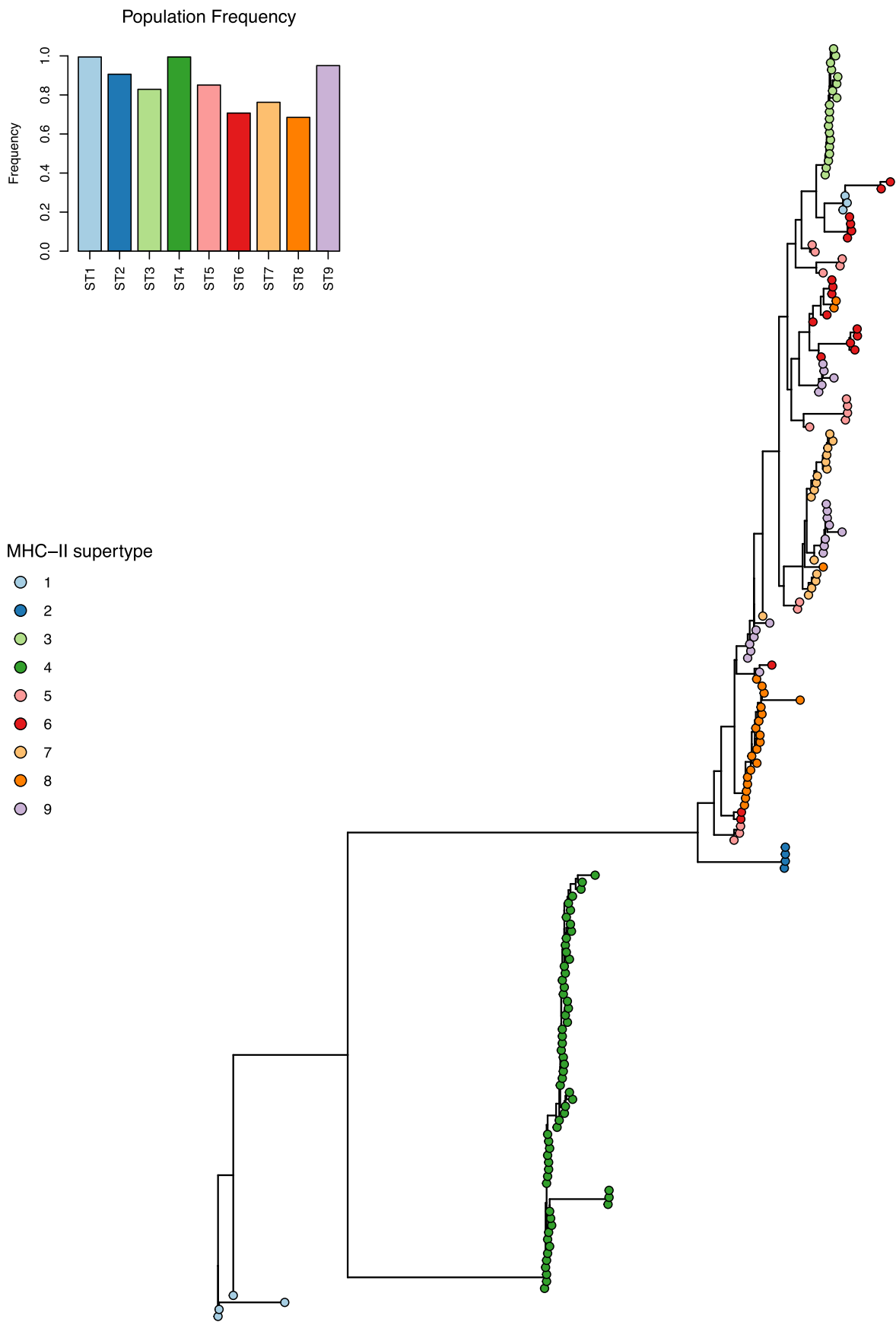
#### 4 | Discussion

This study thoroughly investigated the effects of MHC class II variation on two key components of individual fitness, survival and LRS, using a migratory passerine as a model species while controlling for confounding factors such as temporal environmental heterogeneity, cohort effects and neutral genetic diversity. We found that three out of nine MHC-II supertypes (i.e. clusters of alleles with similar physicochemical properties at their antigen-binding sites) were associated with different components of individual fitness, independent of neutral genetic diversity. Specifically, lifetime offspring production was lower in individuals harbouring supertypes 3 or 7, while individuals with supertype 5 had a higher probability of survival. Overall, the association between specific supertypes and fitness, coupled with the absence of rare supertypes in the population, align with the notion that fluctuating selection, rather than selection for maximum individual diversity, optimal diversity or rare-allele advantage, is a pivotal mechanism maintaining the diversity of MHC-II supertypes in the study population.

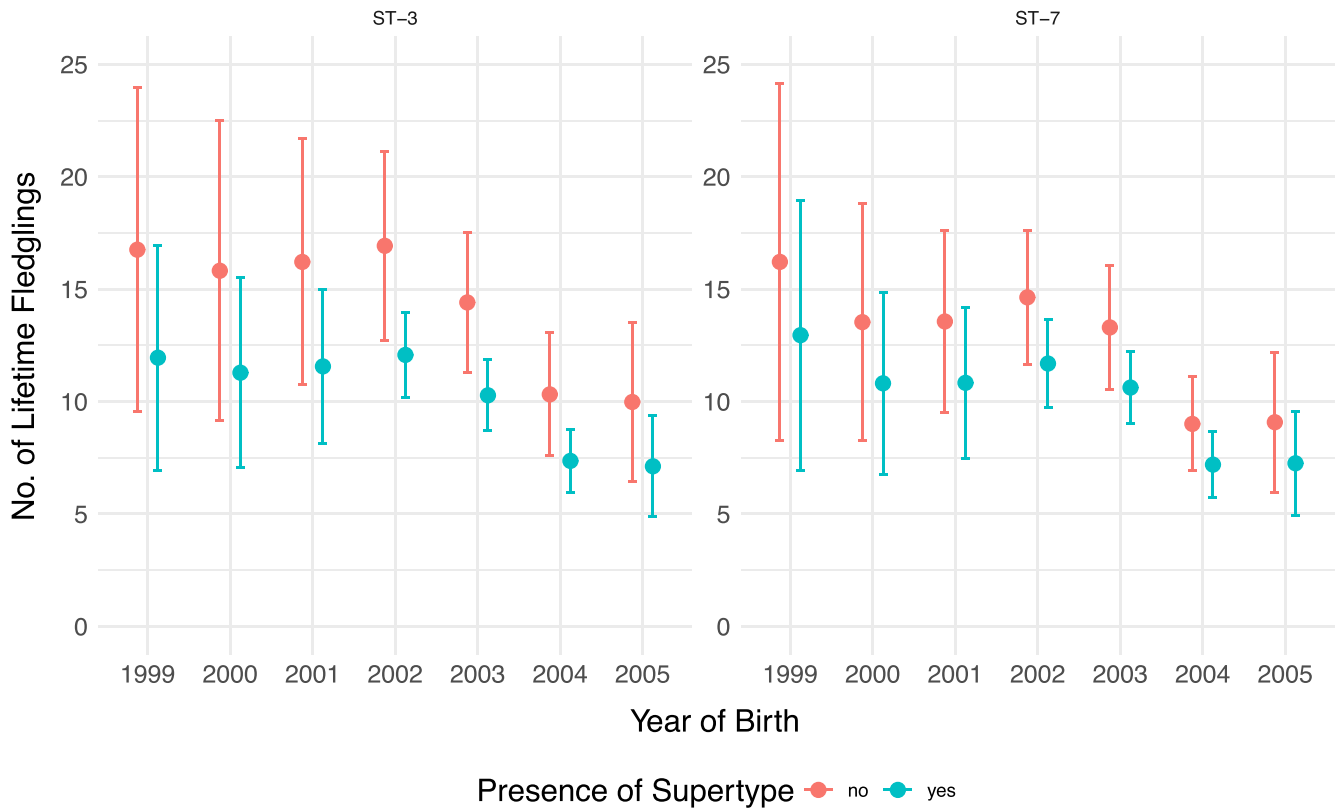
While several works show that MHC diversity affects individual fitness, with high (Doherty and Zinkernagel 1975; Radwan et al. 2012; Thoß et al. 2011) or intermediate (Bonnaud

et al. 2004; Madsen and Ujvari 2006; Kalbe et al. 2009) levels of MHC diversity often associated with higher fitness (but see Roved et al. 2018), we found that overall MHC diversity, in either its linear (*maximal diversity hypothesis*) or quadratic form (*optimal diversity hypothesis*), is unrelated to the survival or LRS of individuals. In contrast, we found that specific functional variants (ST3, ST7 and ST5) underlie the MHC-mediated effects on fitness. Specific MHC supertypes have previously been associated with resistance or susceptibility to infection in a broad range of vertebrate species, including humans *Homo sapiens* (Trachtenberg et al. 2003), fat-tailed dwarf lemurs *Cheirogaleus medius* (Schwensow et al. 2007), *Hipposideros* bats (Schmid et al. 2023), red deer *Cervus elaphus* (Buczek et al. 2016), common frogs *Rana temporaria* (Teacher, Garner, and Nichols 2009), great tits *Parus major* (Sepil et al. 2013), Galápagos mockingbirds *Mimus* (Vlček and Štefka 2020) and Australian tawny dragon lizards *Ctenophorus decresii* (Hacking et al. 2018). However, few studies have observed associations between specific MHC supertypes and individual fitness (Ferreira et al. 2024; Sepil, Lachish, and Sheldon 2012). The fact that, unlike most previous MHC studies, we simultaneously employed two crucial fitness metrics—lifetime reproductive success and long-term survival—which track individual performance from birth to death, may also help explain discrepancies with earlier research.

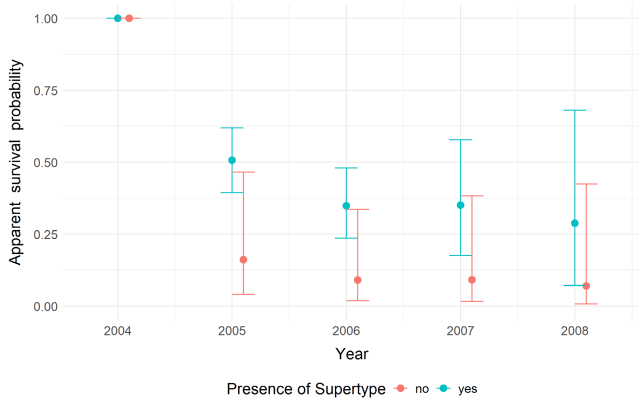
Given the observational nature of this study, we can only speculate on the mechanisms underlying the specific MHC supertype-fitness relationships. For example, differences in fitness-related traits with MHC variation might depend on circumstances experienced in early life, including environmental conditions (Öberg et al. 2015; Reid et al. 2003), parental effort (Alatalo, Gottlander, and Lundberg 1988) and exposure to pathogens with detrimental effects on future reproductive value (Evans et al. 2016). Breeding adults with an inefficient immune response could also be subjected



**FIGURE 1** | (a) Frequencies of the MHC-II supertypes in the sample of pied flycatchers ( $n = 99$ ) used for survival and lifetime reproductive success analyses. (b) Unrooted GTR tree of the 182 MHC-II exon 2 alleles in our dataset based on the full-length DNA sequences. Association of alleles with MHC-II supertypes is indicated with coloured circles.



**FIGURE 2** | Effect of specific MHC class II superotypes 3 and 7 on lifetime offspring production of pied flycatchers (partial effect with 95% CI).



**FIGURE 3** | Effect of specific MHC class II superotypes 5 on apparent survival of pied flycatchers (partial effect with 95% CI). Note that the year refers to the breeding year and not the year of birth.

to (chronic) infections and thus have smaller clutches and/or reduced parental care with a subsequent negative impact on reproductive success (Knowles, Palinauskas, and Sheldon 2010; Oppliger, Christe, and Richner 1996; Raberg et al. 2000; Richner, Christe, and Oppliger 1995; Siikamäki et al. 1997). It is also possible that differences in survival arise if parasitised/infected individuals cannot withstand the physiological stress and energetic demands of major life-history events, such as migration (Altizer, Bartel, and Han 2011), or because of impaired ability to escape from predators (Garamszegi et al. 2015).

Our results indicate that different functional MHC variants are related to different fitness components, which could be

explained by several factors. For example, superotype 5 had a positive effect on survival, suggesting it confers a fitness advantage to carriers. However, ST5 was not associated with an increased LRS, as might be expected if its possession extended the breeding lifespan. A possible explanation is that ST5 functions as a tolerance superotype, providing quantitative resistance that allows hosts to tolerate an infection rather than succumb to it (Westerdahl et al. 2012), though the costs (e.g. decreased resistance) would outweigh the fitness benefits of breeding for more years (Knowles, Palinauskas, and Sheldon 2010; Raberg et al. 2000). Conversely, superotypes 3 and 7 were associated with reduced LRS, but not survival rates. This suggests that ST3 and ST7 may be susceptibility superotypes that pathogens have adapted to. Similarly, a susceptibility MHC superotype was previously found in a wild population of great tits, in which the authors speculated that a reduction in LRS might be a cost of parasite infection induced by a reduction in parental reproductive effort (Sepil, Lachish, and Sheldon 2012). It is also possible that ST3 and ST7, rather than being susceptibility superotypes, provide quantitative resistance and tolerance to infection during earlier life stages, such as the nestling or juvenile phases, which were not examined in our study. If this is the case, we might not detect increased mortality from acute infections in individuals not carrying these superotypes. Still, this scenario is unlikely as we would expect to find the observed reduction in LRS, reflecting a cost of chronic infection (Knowles, Palinauskas, and Sheldon 2010). Lastly, it is possible that a given superotype might confer resistance to pathogens affecting, exclusively or disproportionately, one fitness component. For example, pathogens transmitted outside the breeding season may affect the physical condition of individuals during migration and reduce their

survival probabilities (Risely, Klaassen, and Hoye 2017; van Gils et al. 2007). It is also important to acknowledge that limitations in statistical power may have obscured a statistically significant effect of specific supertypes on all fitness components (i.e. ST3 and ST7 on survival and ST5 on LRS).

Interestingly, we found six out of nine MHC-II supertypes (ST1 and ST5–ST9) comprising alleles from different clades in our phylogenetic analysis of the full-length DNA sequences. This polyphyletic pattern suggests that these supertypes are unlikely to originate from individual common ancestors, but each one may instead have arisen from several ancestral alleles that parallelly evolved similar physicochemical properties in their antigen-binding sites. The remaining three supertypes (ST2–ST4) comprised alleles that formed monophyletic clades in the phylogenetic analysis, suggesting a common ancestral allele for each of these supertypes. Our observations are similar to the findings of a previous study on another migratory passerine, the great reed warbler *Acrocephalus arundinaceus* (Roved et al. 2022), suggesting that positive selection and convergent evolution may both be driving mechanisms that maintain the diversity of MHC supertypes in wild passerines. Lighten et al. (2017) proposed that MHC supertypes are maintained as balanced polymorphisms in epitope/paratope space, while their constituent alleles are expected to follow the dynamics of a Red Queen arms race. Given that MHC genes are tightly linked in haplotypes (Roved et al. 2022), we find it likely that convergent evolution on the antigen-binding properties of MHC alleles should occur when MHC haplotypes—the most immediate currency of selection—experience uniform selection pressures from pathogens. Such a mechanism may well underlie the polyphyletic distribution of the supertypes ST1 and ST5–ST9. We encourage further studies to unravel these patterns, including the effects of pathogen diversity, rates of pathogen evolution, and other potentially impacting factors such as sexual selection and introgression (Ejmsmond and Radwan 2015; Gaczorek et al. 2023; Leclaire et al. 2017; Mingju et al. 2021; Voinescu et al. 2024).

The absence of rare MHC-II supertypes argues against rare-allele advantage as a mechanism driving balancing selection on the MHC class II in our study population. Instead, the divergent effects of specific MHC-II supertypes on fitness agree with the predictions of the *fluctuating selection hypothesis* as a primary driving mechanism. This hypothesis posits that changing environmental conditions influence MHC variation by altering the prevalence of different pathogens and, hence, selection pressures on host immunity over time and space (Awadi et al. 2018). Since the *fluctuating selection* is driven by environmental factors rather than a co-evolutionary arms race between hosts and pathogens, a relationship between the fitness effects and frequency of each MHC supertype is not expected, as observed in this study (reviewed in Radwan et al. 2020). However, given that our study provides a snapshot of the population, we could not critically test the *fluctuating selection hypothesis*. Extending our study over longer time periods and across multiple sites, as well as testing whether MHC variation is associated with specific environmental factors and/or pathogens, would allow further investigations of *fluctuating selection* as a driver of balancing selection on the MHC class II in pied flycatchers (Radwan et al. 2020).

In conclusion, our findings indicate that specific MHC supertypes mediate individual variation in LRS (ST3 and 7) and survival (ST5) in pied flycatchers but suggest no effect of MHC diversity on individual fitness. The supertype-specific effects, the absence of rare supertypes, and a consequent lack of a frequency-dependent relationship between supertypes and fitness, align with the expectations of the *fluctuating selection hypothesis*. Many interesting avenues remain to be explored, such as identifying the potentially specific pathogen(s) underlying the between-individual variation in fitness related to supertypes 3, 5 and 7. It would also be of particular interest to further investigate the importance of *fluctuating selection* in maintaining MHC class II diversity of pied flycatchers by extending the present study over longer periods and including more populations. Given the scarcity of long-term ecological studies on individuals in their natural environments, our results contribute rare and valuable evidence of the importance of MHC variation in wild animal populations.

### Author Contributions

D.C., J.P. and S.S. conceived and designed the study. D.C., C.C. and J.P. collected the data. D.C. conducted the laboratory work. J.R. performed the bioinformatic analyses on the MHC sequencing data, phylogenetic models, tests for positive selection and inference of MHC supertypes, while S.S. and D.C. performed the reproductive and survival analyses. D.C. and J.R. wrote the manuscript in collaboration, with significant input from the co-authors.

### Conflicts of Interest

The authors declare no conflicts of interest.

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### Data Availability Statement

The data that support the findings of this study are available from Dryad, doi: [10.5061/dryad.0p2ngf297](https://doi.org/10.5061/dryad.0p2ngf297), as well as from the authors.

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### Supporting Information

Additional supporting information can be found online in the Supporting Information section.