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MHC-mediated sexual selection on birdsong: Generic polymorphism, particular alleles and acoustic signals

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Abstract

Several hypotheses predict that the major histocompatibility complex (MHC) drives mating preference in females. Olfactory, colour or morphological traits are often found as reliable signals of the MHC profile, but the role of avian song mediating MHC-based female choice remains largely unexplored. We investigated the relationship between several MHC and acoustic features in the collared flycatcher (*Ficedula albicollis*), a European passerine with complex songs. We screened a fragment of the class IIB second exon of the MHC molecule, of which individuals harbour 4–15 alleles, while considerable sequence diversity is maintained at the population level. To make statistical inferences from a large number of comparisons, we adopted both null-hypothesis testing and effect size framework in combination with randomization procedures. After controlling for potential confounding factors, neither MHC allelic diversity nor the presence of particular alleles was associated remarkably with the investigated qualitative and quantitative song traits. Furthermore, genetic similarity among males based on MHC sequences was not reflected by the similarity in their song based on syllable content. Overall, these results suggest that the relationship between features of song and the allelic composition and diversity of MHC is not strong in the studied species. However, a biologically motivated analysis revealed that individuals that harbour an MHC allele that impairs survival perform songs with broader frequency range. This finding suggests that certain aspects of the song may bear reliable information concerning the MHC profile of the individuals, which can be used by females to optimize mate choice.

KEYWORDS
birdsong, Hamilton and Zuk hypothesis, immunogenetics, major histocompatibility complex, parasite-mediated sexual selection, repertoire size

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1 | INTRODUCTION

Several studies in molecular ecology suggest that female preference in many animals is mediated by the composition of the major histocompatibility gene complex (MHC; reviews in, e.g., Bernatchez & Landry, 2003; Kamiya, O'Dwyer, Westerdahl, Senior, & Nakagawa, 2014; Milinski, 2006; Tregenza & Wedell, 2000). MHC encodes crucial molecules for self- and non-self-recognition, which bind antigen-derived peptides and form a complex with T cells, thereby triggering an adaptive immune response (Murphy & Weaver, 2017). Hence, females preferring males with particular MHC characteristics can acquire genetic benefits for their offspring due to its functional role in resistance against many pathogens.

MHC-mediated mating preference may be realized through, at least, three distinct mechanisms that rely on different MHC characteristics and offer different types of benefits (Piertney & Oliver, 2006). First, the advantage may be manifested via the effect of particular alleles, as new or rare alleles can be effective against parasite strains that quickly co-evolve with host defence mechanisms (Bonneaud, Perez-Tris, Federici, Chastel, & Sorci, 2006; Sin et al., 2014; Westerdahl, Asghar, Hasselquist, & Bensch, 2012). Under this scenario, the host–parasite evolutionary cycles can promote negative frequency-dependent selection, which generates polymorphic MHC alleles within the host population. Second, absolute MHC diversity or heterozygosity can also serve as a basis for female mate choice. Preference for partners harbouring diverse and/or heterozygote MHC alleles should incur benefits due to increased resistance to parasites, because such individuals can more effectively cope with a wider array of pathogens (Brouwer et al., 2010; Oliver, Telfer, & Piertney, 2009; Worley et al., 2010). Mechanisms for MHC allele number and heterozygosity can be regarded as the two sides of the same coin, because a large number of alleles also implies high heterozygosity and preference for such traits incurs direct and indirect benefits in terms of immunocompetence (Kamiya et al., 2014; Landry, Garant, Duchesne, & Bernatchez, 2001). Third, mate choice based on genetic compatibility can be in effect, if females choose partners relative to their own MHC composition; for example, reproducing with males with dissimilar MHC profile can lead to increased MHC diversity and heterozygosity in the offspring (Juola & Dearborn, 2012; Schwensow, Eberle, & Sommer, 2008; Tregenza & Wedell, 2000).

For MHC-mediated mating preference to work, it is required that males expose traits that reliably indicate some attributes of the MHC composition (Bernatchez & Landry, 2003; Milinski, 2006; Penn & Potts, 1999). If MHC genotype covaries with some perceptible phenotypic trait, females can rely on such signals in their optimal mating decisions. Proximate mechanisms functioning in MHC-mediated mate choice typically include odour signals in many animals, as MHC molecules can be bound to volatile chemicals that are excreted via the skin, urine or faeces thus can contribute to body odours (Carroll, Penn, & Potts, 2002; Milinski, Croy, Hummel, & Boehm, 2013; Milinski et al., 2005; Olsson et al., 2003; Penn, 2002). In birds, chemical signalling is still poorly recognized, but the secretion of the uropygial gland or feather microflora have recently been implicated as a potential source of scent cue for MHC signalling (Leclaire et al., 2014; Slade et al., 2016; Strandh et al., 2012), while olfactory perception has also been described at least in some species (Fracasso, Tuliozi, Hoi, & Griggio, 2018; Griggio, Fracasso, Mahr, & Hoi, 2016). In animals that only partially rely on olfactory cues, condition-dependent visual or behavioural traits can signal genetic resistance, because only males with high immunocompetence (as determined by their MHC genotype) can bear the costs of elaborated sexual displays (Folstad & Karter, 1992; Hamilton & Zuk, 1982). Accordingly, several studies in different taxa have shown that the expression of showy displays in males is related to MHC allelic diversity, heterozygosity or the presence of particular alleles or allele combinations (Ditchkoff, Lochmiller, Masters, Hoofer, & Van Den Bussche, 2001; Dunn, Bollmer, Freeman-Gallant, & Whittingham, 2013; Griggio, Biard, Penn, & Hoi, 2011; von Schantz, Wittzell, Goransson, & Grahn, 1997; Setchell, Charpentier, Abbott, Wickings, & Knapp, 2009; Whittingham, Freeman-Gallant, Taff, & Dunn, 2015).

Although avian song is one of the most famous models of sexual selection (Catchpole & Slater, 2008; Searcy & Nowicki, 2000), acoustic traits in birds have not typically been investigated as potential signals of MHC characteristics (Slade, Watson, & MacDougall-Shackleton, 2017). This is surprising, because birdsong is very complex, and is often subject to parasite-mediated sexual selection (review in Garamszegi, 2005), and one can envision several ways by which it enables MHC-mediated female choice. For example, song displays include several condition-dependent traits that can potentially operate as handicap signals (Hamilton & Zuk, 1982; Vehrencamp, 2000). If such mechanism is at work, only immunocompetent males with the appropriate genetic background of the MHC can pose costly song traits. Furthermore, through the content of songs (i.e., syllable composition), individual repertoires can hold information on the singer's qualitative attributes (Gil & Gahr, 2002). Such a signalling can be useful if, for example, disassortative mating occurs, that is, when females make their choice based on male attributes relative to their own. Furthermore, similarly to complex odour signals in mammals, avian songs can code an enormous amount of information due to the fact that songs contain a unique combination of song elements (Smith, 2014). Therefore, it may be hypothesized that compositional traits of songs reveal aspects of the allelic composition at the MHC region, with certain song elements being nonrandomly associated with certain MHC alleles.

This mode of signalling would require that MHC characteristics are mechanistically linked to the production of the signals in a similar way to volatile components of olfactory cues. For birdsong, such direct evidence is missing, but some indirect facts are suggestive. For example, in a rodent model, the development and plasticity of the central nervous system functionally require MHC class I derivatives (Huh et al., 2000), and such mechanisms may also be in effect in the regions of the avian brain that govern song learning and production. Neuropeptides may also play a mediator role, as substance

P, which is a pro-inflammatory factor that can enhance MHC expression (Annunziata, Cioni, Santonini, & Paccagnini, 2002; McCluskey & Lampson, 2001), has been found to accumulate in the song control nuclei in the brain, especially in species with large repertoire size (Li, Zeng, Zhang, & Zuo, 2006). Moreover, MHC exhibits a complex assembly with several genes that are spanned across at least four chromosomes (Balakrishnan et al., 2010; Ekblom et al., 2011), which facilitates genetic linkage with genes regulating the development and functioning of the song control nuclei in the brain that are also dispersed in the avian genome (Lovell, Clayton, Replogle, & Mello, 2008). Finally, a relationship between song traits and MHC profile may also operate via an indirect link, such as through condition dependence. For example, if particular MHC alleles lead to resistance against debilitating pathogens, parasite-free individuals harbouring these alleles will be in generally better condition. Then, if better condition permits individuals to use, for example, syllables that are energetically costly to produce (see Suthers, Vallet, & Kreutzer, 2012 as an example), the presence of resistance alleles will increase the probability of these costly syllables to be sung.

The collared flycatcher (*Ficedula albicollis*) is a European passerine that exhibits an elaborate singing behaviour during courtship. The song performance of males consists of songs that are few seconds long and built of syllables of different types, with individual males being able to produce up to 50 syllable types with a moderate overlap with the repertoire of other males (e.g., Garamszegi, Zsebők, & Török, 2012; Garamszegi et al., 2007). Certain song traits, such as song rate and song duration, may signal health status, and correlative evidence suggests that the same attributes of song may also convey mating benefits in terms of quick pairing (Garamszegi, Merino, Török, Eens, & Martínez, 2006; Garamszegi et al., 2004). In the closely related pied flycatcher (*Ficedula hypoleuca*), experimental evidence shows that males with large repertoire size are more attractive for females than males with small repertoire size (Lampe & Sætre, 1995). Concerning the role of individual-specific song composition, it is known in the collared flycatcher that males with similar song content enjoy similar mating success, while male–male competition can induce males from adjacent territories to produce songs with relatively little overlap in their repertoires (Garamszegi et al., 2012). Therefore, both the quantitative and qualitative aspects of songs in this species are likely shaped by sexual selection.

In this study, we investigated the possibility that qualitative or quantitative aspects of song production in the collared flycatcher can be associated with the MHC profile of the singer, thereby fulfilling prerequisites for MHC-mediated female preference for certain song traits. To develop a complete understanding of how songs may function as signals of genetic quality, we undertook an extensive song analysis and screened MHC class IIB. Given that signal design may determine the information being conveyed and different signal types may be involved in signalling, we distinguished between quantitative (i.e., song duration, song rate) and qualitative song traits (e.g., syllable composition). As for the genetic analyses, we specified the allelic composition at the MHC by amplifying a 197-bp fragment of the class IIB second exon of the molecule, which is related to the peptide-binding region. In a correlative design, we estimated the allelic composition and diversity of the individuals and related these traits to the derived song variables. We investigated the potential mechanisms involved in MHC-mediated female preference based on the predictions presented in Table 1.

TABLE 1 The predictions concerning the relationship between characteristics of birdsong and MHC. (i) Hypotheses assuming female preference for allelic diversity (or heterozygosity) predict that the absolute number of alleles (or its quadratic term if not large, but intermediate numbers of alleles convey the most benefits; sensu Kalbe et al., 2009) is associated with quantitative (i.e., repertoire size, song duration or song rate) or qualitative (i.e., the presence of particular syllable types in the song) song traits. (ii) Models assuming preference for particular alleles predict that the individuals harbouring particular alleles that offer protection (or susceptibility) to pathogens or have other consequences for fitness perform songs of higher quality as reflected by a quantitative or qualitative trait. (iii) Hypotheses emphasizing female preference for major histocompatibility complex (MHC) compatibility predict that relative and not absolute trait elaboration is important; thus, males with similar song composition would have similar allelic composition

	Song		
	Quantitative song traits	**Presence of particular syllables**	**Similarity in song composition**
MHC			
Allele number	Female preference for allelic diversity	Female preference for allelic diversity	
Presence of particular alleles	Female preference for particular alleles	Female preference for particular alleles	
Similarity in allelic composition			Female preference for MHC compatibility

2 | METHODS

2.1 | Field procedures

Our fieldwork was carried out in the Pilis-Visegrádi Mountains in Hungary (47°43′N, 19°01′E), where breeding plots involving more than 800 nest boxes have been established for the long-term study of hole-nesting passerines (Török & Tóth, 1988), wherein the collared flycatcher is a common breeder. Data to test the predictions of this study were collected during the field seasons of 3 years (2007–2009), but occasionally data from other field seasons have been incorporated (see below).

As a part of our standard field protocols established in 1999, we made song recordings as follows. During the course of the season, we regularly visited the field site to detect unpaired males showing the typical courtship behaviour on their territory during the most active

morning period (usually between 6.00 and 12.00 hr). Then, after stimulating them to sing with a decoy female, we recorded their songs with a Sennheiser ME62 microphone attached to a Telinga parabolic dish and with a MicroTrack II digital sound recorder. From each male, we obtained at least 20 songs (see reasoning in Garamszegi et al., 2002), and at least 10 min of recording to allow an accurately comparable estimate of song traits. We achieved the following sample sizes of males for which we obtained recordings with sufficient songs and that were then successfully captured for identification (see below): $N_{2007} = 25$, $N_{2008} = 12$ and $N_{2009} = 41$. We could extend this initial data set with data from other years, because for $N = 12$ males that have been sampled for MHC genotyping (see below), song recording became available in previous or proceeding years (we apply the same song recording and analysis standards in each year).

When the song recordings were completed, we aimed at capturing the birds using a conventional nest-box trap, which was successful in 84.6% of efforts. To verify that our sample relying on captured males was unbiased, we compared the song variables between the groups of captured and noncaptured birds. These comparisons revealed no statistical evidence for biased sampling due to between-individual differences in trappability (all $ps > 0.31$ accounting for multiple testing). Captured birds were marked with metal rings with unique numbers for identification, permitting us to confirm that each male was included in our sample only once. Upon handling, we performed the standard morphological measurements (data not used here) and classified birds as adults or juveniles (based on their plumage colour). On the basis of our standardized ringing regimes and the capture–recapture history of individuals, we could assign them into a minimum age category (i.e., being at least in their 1st–6th year of life) that was subsequently used as a continuous estimate of age. Blood samples were taken from all individuals by puncturing the brachial vein and stored in ethanol for subsequent molecular analyses. All birds were released after these protocols. The date of the recording was noted to control for potential date effects in the statistical models.

2.2 | Song analysis

From the raw recordings, we manually selected 20 consecutive songs of good quality (i.e., with a low level of background noise and in which no other birds interacting) submitted to further spectrographic analyses. Songs are 2- to 3-sec-long features consisting of uninterrupted series of acoustic elements, so-called syllables (10.13 ± 5.15 [mean ± *SD*] syllables per song; see spectrograms in Garamszegi et al., 2002, 2007, 2012).

Before submitting the selected songs to automatic measurements of frequency contours, the start and the end points as well as the minimum and maximum frequency of each syllable were marked manually on the computer's screen. Then, using a self-written Matlab program, we measured the following independent variables on the level of songs: song duration (s), tempo (the ratio between the number of syllables within song and song duration, 1/s), frequency band of songs (the difference between the highest and lowest frequencies, Hz), mean frequency (Hz) and short-term complexity (number of different syllable types/total number of syllables within songs). On a higher organizational level (song sequences that consisted of 20 consecutive songs reflecting song performance), we defined two variables: song rate (the number of analysed songs divided by the length of the corresponding recording, 1/min) and repertoire size (the number of different syllable types within the sample of 20 songs). Hereafter, we refer to these variables as quantitative traits, which could be derived for all of the 90 males with song recording. Our previous studies revealed that when recordings for the same males were made on different days, song traits quantified along the above sampling regime have considerable within-individual repeatability (Garamszegi et al., 2004; Zsebők et al., 2017).

For the qualitative comparison of individuals based on their syllable composition, we used another Matlab program, which assigns syllables into distinct categories based on a computer-aided manual classification algorithm (for details see Garamszegi et al., 2012). Briefly, the program automatically measures several frequency and temporal parameters on each syllable; then, based on these measurements, it provides a suggestion for syllable categorization. However, it always requests a final decision from the investigator for the assignment of syllables into a particular group. This coding process allowed us to create a universal syllable library, in which syllable codes are collectively applicable to and qualitatively comparable among all individuals. On the basis of this methodology, we determined the list of syllables that particular males incorporated in their repertoires, and these sets were subsequently used for the qualitative comparison of songs (see below). The quality of the recordings permitted the full categorization of syllables according to the universal library only for a set of 84 individuals using 1,083 syllable types altogether. Hence, analyses focusing on the qualitative aspect of songs and referring to the presence of particular syllables in the song correspond to this sample size. The syllable composition of individuals, as assessed based on 20 songs, is also a highly repeatable trait even in the between-year context ($R > 0.65$; L. Z. Garamszegi & S. Zsebők unpublished data). The Matlab programs used for the sound analyses are available at https://github.com/zsebok/Ficedula.

To calculate the pairwise distances between males based on the content of their songs, we applied procedures from Garamszegi et al. (2012). Briefly, for each pair of individuals we obtained a similarity index based on the overlap of their repertoires (proportion of syllable types that they have in common) after accounting for the relative occurrence of syllables in their songs. This index varies from 0 to 1, with a score of 0 indicating that the two males build their songs from completely different syllables, and with a score of 1 reflecting a complete overlap in their song content and the frequency of syllable use. To obtain distance statistics from these similarity scores, we systematically subtracted the calculated similarity indices from 1.

2.3 | Molecular methods for MHC class II genotyping

A total of 123 different males from the three focal years (2007–2009) were successfully screened at their MHC at a strict criterion for

coverage (see protocols below). This sample included individuals, for which we had not obtained song recording during the study years. Hence, the final data set that could be used to test our predictions included 90 males, for which we had information on both MHC and song.

2.3.1 | Amplification and 454 sequencing

We used 454 pyrosequencing to amplify 197-bp fragment of MHC class IIB second exon of the collared flycatcher. The species-specific forward (FicL1938) and reverse (FicR1938) primers incorporated to standard fusion primers were employed to obtain fragments of interest (Zagalska-Neubauer et al., 2010). PCR amplification was performed in final volume of 20 μl containing 0.2 mM of each dNTP, 1 μM of each primer, 25 mM $MgCl_2$, 1 U of Taq polymerase (Fermentas), 2 μl of 10× PCR buffer with $(NH_4)_2SO_4$ and approximately 100 ng of genomic DNA. Cycling programs consisted of 3-min initial denaturation at 94°C, followed by 33 cycles at 94°C for 30 s, 58°C for 30 s, 72°C for 30 s and final extension at 72°C for 3 min. Amplicon concentration was estimated on agarose gels, measured on NanoDrop, pooled into approximately equimolar quantities and finally purified with the MinElute PCR Purification Kit (Qiagen). Obtained pools were sequenced as a part of a single 454 Titanium technology run and individuals tagged with particular MID. Data were extracted and analysed with jMHC software (Stuglik, Radwan, & Babik, 2011). The output was further analysed with use of GENEIOUS v. 5.6.5 (Biomatters 2012), MEGA 5.1 and BIOEDIT (Hall, 1999).

2.3.2 | MHC genotyping and distinguishing true alleles from artefacts

We have used a stepwise procedure to filter out artefacts from real alleles generated by high-throughput sequencing technologies (Babik, Taberlet, Ejsmond, & Radwan, 2009). The detailed description of our MHC protocol could be found in Zagalska-Neubauer et al. (2010).

First, we excluded individual with low coverage (<200) and variants containing indels and/or presented in the data set in just one or two copies. Then, we calculated the maximum per-amplicon frequency (MAPF) of each sequence variant and sorted the variants according to MAPF criterion. Variants above 2.5% can be considered to offer a satisfactory coverage and meet the primary criterion of putative true alleles. Variants with MAPFs between 1.5% and 2.4% were carefully sieved as could contain high number of artefacts, whereas nearly all variants from 0.5% were regarded as artefacts.

The software GENEIOUS (Biomatters 2012) was used as an additional approach to examine putative alleles and the existence of chimeras originated by in vitro recombination between true alleles (Galan, Guivier, Caraux, Charbonnel, & Cosson, 2010; Lenz & Becker, 2008). When grouping the sequences within individuals by similarity, chimeras could be easily detected because they typically appear in a low number between the two clusters of sequences corresponding to the putative alleles from which they are originated. Further, sequences were translated to confirm that they are apparently functional (e.g., without stop codons or frameshifts) and inspected by eye to verify that the variants found in the polymorphic sites were also presented in other alleles from the population. Finally, we identified 90 true alleles in the processed sample. Note that by allele, we refer to a fragment of the exon 2 MHC class II for convenience.

We have previously shown that our genotyping approach is reliable as i) there were no discrepancy between the genotypes of 28 individuals amplified in two sets of independent and blind analyses (i.e., repeatability is $R = 1$) and ii) coverage and number of alleles found in an individual were unrelated (see Garamszegi et al., 2015). However, when working with high-throughput sequencing approaches, one may assume that the existence of artefacts cannot be completely ruled out. However, it is reasonable to assume that individuals with more elaborate song traits are not more likely to generate artefacts during NGS than individuals with less elaborate song traits and, thus, that our conclusions are unbiased.

We estimated the evolutionary divergence over pairs of the translated full nucleotide sequences by considering the entire allele repertoire of individuals. We calculated the number of amino acid differences per site by considering all sequence pairs between males in MEGA 7 (Kumar, Stecher, & Tamura, 2016). This analysis involved 1,220 amino acid sequences. These between-individual differences were tabulated into a matrix format for further analyses.

2.4 | Statistical analyses

2.4.1 | The relationship between quantitative song traits and the number of MHC alleles

To analyse the relationship between quantitative song variables one by one (e.g., song duration, tempo, song rate, repertoire size) and MHC allelic diversity (prediction 1 in Table 1), we built general linear mixed models (GLMMs) with the focal song variable as a response and MHC allele number as a predictor by assuming a Gaussian error distribution. We also built models to which we added the quadratic term for MHC allele number, because some theoretical models predict that due to the autoimmune side effects, allele numbers are not maximized but optimized at the intermediate level (Kalbe et al., 2009; Slade et al., 2017; Wegner, Kalbe, Kurtz, Reusch, & Milinski, 2003). The models also included continuous measure of male age and date of the recording as covariates to control for their confounding effects on song (Garamszegi et al., 2004, 2007). The date of recording was standardized among years by defining day 1 in each year based on the date when the first male was successfully recorded. Year effects were also considered by entering this variable as a random factor in the model. We repeated these modelling exercises for each song trait. Parameter estimates from the fitted models were obtained by fitting models using maximum likelihood rather than restricted maximum likelihood (Bolker et al., 2009). For interpretations, p values were adjusted for multiple testing (see below).

During the course of the statistical analyses, before interpreting any model outcome, we routinely performed numerous model diagnostics statistics to avoid misleading results based on statistical

artefacts (Loy & Hofmann, 2013). These checks included the investigation of the distribution of residuals, issues about multicollinearity and the effect of influential data points. To meet statistical assumptions, the number of MHC alleles and song duration were $\log_{10}$-transformed, while other variables were left untransformed. After these transformations, the diagnostics analyses did not show obvious deviations from the assumptions of linear models.

2.4.2 | The relationships between particular syllables and the number of MHC alleles

We also investigated that instead of quantitative song traits, the presence of a particular syllable type in the repertoire as a qualitative song trait indicates superior immune defence due to higher MHC allelic diversity. We went through each syllable type and compared the number of MHC alleles between males in which we did and in which we did not detect the given syllable type. To disregard very rare and very common syllables and avoid meaningless comparisons, we only considered those syllables types that were detected in at least 10% but not more than in 90% of all individuals (resulting in $N = 110$ syllable types). Accordingly, for each syllable type considered, we fitted mixed models with binominal error distribution, in which syllable presence (bivariate state "yes"/"no") was the response. The predictive part of the model was the same as above for the quantitative song variables including sets with and without the quadratic term for MHC allele number. The only addition was that we also entered repertoire size along with the list of predictors to control for overall syllable diversity (as more diverse individuals are more likely to have a specific syllable). We extracted the parameter of interest (coefficient for MHC allele number and for its second-order term) and the associated p value.

To make inferences from this large number of outputs, we applied three approaches each being loaded with different benefits and shortcomings. First, we assessed the significance of particular effects by applying a control for the number of test performed under the same hypothesis. For this control, we chose the correction method that is based on false discovery rates (FDR; Benjamini & Hochberg, 1995) for reasons detailed in Pike (2011). Given that we evaluated 110 models, the nominal significance level decreased to $p < 0.000454$ after the above correction for each syllable, which raised a considerable loss of power ($\beta = 0.232$, when considering $r = 0.3$ medium effect size). Therefore, the inference based on p values permits detecting few, if any, strong (i.e., $r > 0.44$ at $\beta = 0.8$ power) associations between the presence of a particular syllable and MHC allele number. Second, we also investigated the distribution of effect sizes (unstandardized regression coefficients that are comparable across models given that each of them relied on the same sample size and design) over a random expectation. The distribution of the random expectation was determined based on the random reshuffling of the vector of presence/absence of syllables across individuals. This data shuffling was repeated 1,000 times, and from these randomization results, we extracted the distribution of mean effect sizes under the null hypothesis of no association between traits. We compared the distributions of the mean effect sizes from the shuffled and nonshuffled, real data set. Such an inference based on mean effect sizes can reveal whether several syllables are concordantly associated with allele number, at least with small or intermediate effect size. Note that we also considered applying an information-theoretic approach (e.g., sensu Lukasch et al., 2017), but finally we found it inappropriate in our design and data.

The two above approaches applied do not allow capturing weak/intermediate associations that are only restricted to a handful of syllable types or that vary in their direction. Therefore, as a third approach, we chose an inference based on biological rather than statistical concepts. Therefore, we identified syllables that are potentially relevant in female preference based on their relationship with pairing latency. Pairing latency can be defined as the number of days that elapsed between arrival (when a given male is first seen in our study area, i.e., the date of recording) and laying date (the date when the first egg is laid later by the male's breeding pair). It reflects mating success, as males that are successful in mate attraction establish pair-bond relatively quickly and thus realize short pairing latency (Buchanan & Catchpole, 1997; Garamszegi et al., 2012; Gil & Slater, 2000). Accordingly, for each syllable, we built a GLMM, in which the pairing latency ($\log_{10}$-transformed) was the response variable and the presence of syllable, repertoire size, standardized date and age were fixed predictors, and year was a random effect. From these models, we extracted standardized effect size (r) for the relationship between the presence of the syllable and pairing latency using the form $r = t/\sqrt{(t^2 + df)}$ (Nakagawa & Cuthill, 2007). We regarded those syllables functioning in female choice, for which we could obtain an $r > 0.3$ (medium effect sizes) from the above model. We could identify eight biologically important syllables in this way (Supporting Information Figure S1), and we re-examined their relationship with MHC diversity.

2.4.3 | The relationship between quantitative song traits and particular alleles

To test for signalling mechanisms mediated by particular alleles (prediction 2 in Table 1), we investigated whether the presence of particular MHC alleles was associated with features of song. For each MHC allele, we fitted a GLMM, in which one quantitative song variable was the response and the bivariate state of the MHC trait (i.e., the given allele is present or absent) was the focal predictor. In addition, we also included standardized date, age and year as the control predictors by applying the same structure as in the models above. We added also the number of MHC alleles to the predictive part, to control for diversity effects. We fitted models for all the quantitative song variables and alleles that were neither too rare nor too common ($10\% < p < 90\%$) to allow meaningful variation in the focal predictor variable. These combinations resulted in 7 song traits × 22 MHC alleles = 154 models, from which we made inferences based on the strategy of the three approaches we detailed above. In the first framework that is based on null-hypothesis testing, we calculated the FDR-corrected p values for each song trait/MHC allele comparison. In this case, the nominal significance level shrank to $p < 0.000325$ leading to a statistical power of $\beta = 0.235$ (at a medium effect size)

per model. For the second inference based on effect sizes, we computed a standardized *r* effect size from the estimated parameters of each model for the focal relationship (song traits and MHC allele) and examined whether the distribution of these effect sizes deviated from the expectations of random chance. This later null distribution was determined as above, that is, based on the random reshuffling of data 1,000 times. The third, biologically motivated interpretation relied on the fitness value of MHC alleles, which was estimated relying on the strength of association between the presence of allele and longevity. If a particular allele implies parasite resistance (or susceptibility), individuals that harbour these alleles should realize higher (or lower) survival and live longer than individuals that do not have the allele (e.g., Bateson et al., 2016; Brouwer et al., 2010; Lukasch et al., 2017; Paterson, Wilson, & Pemberton, 1998; Sepil, Lachish, & Sheldon, 2013). Correspondingly, we tested for the association of each allele with lifespan (estimated as the oldest age in our long-term ringing records) in a *t* test, from which the derived statistic was converted into an effect size (*r*). If this effect size exceeded 0.3 for a particular MHC allele, we considered it to have a biological function. Two alleles surpassed this criterion (Supporting Information Figure S2), so their relationships with song traits were of particular interest.

2.4.4 | The relationship between particular syllables and alleles

To test the hypothesis emphasizing the role of particular alleles, we also explored whether there was any association between the presence of each MHC allele and the presence of each syllable type in the song. Primarily, we considered all possible combinations (22 * 110 = 2,420, when focusing on alleles and syllables with occurrence 10% < *p* < 90% only), but given that many syllables and alleles are relatively rare, the sporadic incidence of these co-occurring in the same individual generates very unbalanced data structure. Therefore, we created GLMMs for syllable and allele combinations only, for which we could identify at least five males harbouring the allele and using the syllable in their repertoire ($N = 550$). In these models, syllable presence was the response and allele presence was the main predictor, while the control variables (age, date, year, repertoire size and number of MHC alleles) were also entered as above. From these models, we extracted *p* values and effect sizes for the focal relationship to make inferences based on the three approaches we described earlier. When applying on null-hypothesis testing, the correction for multiple testing reduced the significance level to $p < 0.000091$, which drastically increased the risk of committing type II errors (statistical power: $\beta = 0.122$). For the inference based on effect sizes, from each model we used the parameter estimate that characterizes the relationship between the presence of allele and that of the syllable (these coefficients were unstandardized as they corresponded to the same sample size). Then, we verified whether the distribution of these effect sizes followed the random expectations based on randomization processes described above. Finally, we re-examined the strength of relationship for alleles and syllable types, for which we had previously ascribed biological role.

2.4.5 | Similarity in song and estimated genetic relationships based on MHC sequences

Finally, we tested whether song composition has a potential to indicate the similarity between males in their MHC profile that would allow females to assess compatibility relative to their own MHC profile (prediction 3 in Table 1). Along this hypothetical scheme, we expected that the distance matrix that depicts the differences between males in their syllable usage would resemble at least to some degree the distance matrix of males that is based on the dissimilarity of their MHC sequences. To test this, we estimated the association between the two distance matrices with a Mantel test.

The statistical analyses were carried out in the statistical environment of R (R Development Core Team 2016). For the mixed modelling, we used the package *glmmADMB* (Skaug, Fournier, Bolker, Magnusson, & Nielsen, 2016). For a part of the model diagnostics, we relied on package *HLMdiag* (Loy & Hofmann, 2014) and the VIF function available in package car (Fox & Weisberg, 2011). Effect sizes were calculated in the package compute.es (AC Del Re 2013).

3 | RESULTS

3.1 | The relationship between song traits and the number of MHC alleles

In models in which we tested whether quantitative traits reflect MHC allelic diversity, we did not find statistical evidence for quantitative traits being related to the number of alleles or to its quadratic term (Table 2, Supporting Information Figure S3). A significant pattern emerged in the model for the mean frequency of songs, in which the number of MHC alleles was a positive predictor (effect size: $r = 0.227$, 95% CI = 0.021–0.415). However, after considering the number of tests performed ($N = 7$), this relationship was not significant anymore ($p = 0.222$).

It also appears that the number of MHC alleles is not signalled via syllable composition, as the effects for the presence of particular syllables in the repertoire did not differ from random expectations (Supporting Information Table S1). Out of the 110 comparisons, the presence of three syllables was associated significantly with MHC allele number and that of two other syllables with the quadratic term of MHC allele number. However, after controlling for multiple testing, *p* values were far from significant ($p > 0.503$). When applying a biological focus, none of the eight syllables that predict mating success (Supporting Information Figure S1) was related significantly to measures of MHC allelic diversity ($p > 0.186$). The mean of the slope for the number of alleles was −0.005 ($SE = 0.017$ across models for different syllable types), while for the quadratic term it was −0.041 ($SE = 0.011$ across models for different syllable types). The randomization procedures revealed that these observed effect sizes fell within the distribution mimicking the null expectation of no association between traits (95% CI calculated across the 1,000 iterations, linear term: −0.030–0.022; quadratic term: −0.052–0.013).

TABLE 2 The relationship between major histocompatibility complex II diversity (the number of alleles) and quantitative song traits in the collared flycatcher. Outputs from mixed models, in which a given song trait was entered as a response and the number of alleles (linear model) and its second-order term (quadratic model) were the focal predictors. Age and standardized date were also entered as fixed effects to control for their potentially confounding effects. Year was used as random effect

	Linear model			Quadratic model		
Predictors	***F***	***df*[a]**	***p***	***F***	***df*[a]**	***p***
Repertoire size						
Number of alleles	0.272	1, 78.52	0.603	1.421	1, 78.48	0.237
(Number of alleles)^2				1.679	1, 78.61	0.199
Age (juvenile/adult)	7.864	1, 78.22	0.006	7.694	1, 77.31	0.007
Date	2.080	1, 17.62	0.167	1.771	1, 18.28	0.200
Song rate						
Number of alleles	0.391	1, 77.78	0.534	0.128	1, 81.54	0.721
(Number of alleles)^2				0.068	1, 8164	0.795
Age (juvenile/adult)	0.390	1, 77.73	0.534	0.414	1, 77.18	0.522
Date	1.318	1, 16.06	0.268	1.311	1, 18.92	0.267
Song duration						
Number of alleles	0.001	1, 83.24	0.975	0.666	1, 84.97	0.417
(Number of alleles)^2				0.695	1, 84.99	0.407
Age (juvenile/adult)	0.951	1, 79.77	0.332	1.024	1, 79.63	0.315
Date	12.483	1, 3.668	0.027	12.318	1, 3.623	0.029
Tempo						
Number of alleles	0.046	1, 82.54	0.831	1.619	1, 82.76	0.207
(Number of alleles)^2				1.573	1, 82.77	0.213
Age (juvenile/adult)	7.130	1, 83.01	0.009	6.750	1, 81.98	0.011
Date	0.351	1, 64.04	0.555	0.414	1, 68.74	0.522
Within-song complexity						
Number of alleles	2.241	1, 85.00	0.138	0.918	1, 84.00	0.341
(Number of alleles)^2				0.522	1, 84.00	0.472
Age (juvenile/adult)	0.039	1, 85.00	0.844	0.057	1, 84.00	0.813
Date	0.779	1, 85.00	0.380	0.989	1, 84.00	0.323
Frequency bandwidth						
Number of alleles	0.891	1, 77.10	0.348	0.003	1, 84.79	0.957
(Number of alleles)^2				0.043	1, 84.81	0.835
Age (juvenile/adult)	0.993	1, 77.58	0.322	1.005	1, 83.56	0.319
Date	1.944	1, 55.24	0.169	1.937	1, 57.60	0.169
Mean frequency						
Number of alleles	4.774	1, 81.04	0.032	1.953	1, 80.88	0.166
(Number of alleles)^2				1.120	1, 80.85	0.293
Age (juvenile/adult)	7.756	1, 81.81	0.007	8.138	1, 80.62	0.006
Date	4.461	1, 84.50	0.037	4.468	1, 83.467	0.038

[a]Satterthwaite approximation.

3.2 | The relationship between song traits and particular alleles

As predicted by the theoretical model operating with particular alleles, we examined whether there was an association between the presence of MHC alleles and the quantitative song variables (Supporting Information Table S2). We found that 12 of the performed comparisons ($N = 154$) reached statistical significance. However, after the appropriate control for multiple testing, none of these associations can be considered as significant ($p > 0.251$). We also made inferences from this set of comparisons based on effect sizes, which revealed that the standardized mean effect size across alleles and song variables was $r = -0.009$ ($SE = 0.003$). This fell within the range that corresponds to the null expectations of the randomization

approach (95% CI calculated across the 1,000 iterations: −0.017–0.016). When we limited our focus to those alleles that predict longevity (Supporting Information Figure S2), we detected a medium effect size ($r = 0.300$, $p = 0.004$; which was within the 2% strongest effects of the 154 comparisons) for the association between allele "1307" and frequency bandwidth. This pattern indicates that males harbouring this allele have a lower probability to survive (Supporting Information Figure S2) and also produce songs with broader frequency range (Figure 1).

We identified 16 significant associations (out of 550 tests) between particular alleles and particular syllables in the song (Supporting Information Table S3). This incidence was within the expectation of random chance (<5%), which was further supported by the FDR-corrected significance values ($p > 0.905$). None of the relationships between syllable types that linked to mating success and between alleles that have consequences for survival was significant ($p > 0.568$). When focusing on effect sizes, we detected that the mean of the model coefficients describing the strength of the association between particular syllables and alleles was larger than zero (mean ± $SE = 0.196 \pm 0.025$). However, positive correlations between traits may emerge because of the effect of relatively rare alleles and syllables, because these are simultaneously absent in many individuals making them to appear associated probabilistically. When we applied randomization procedures, the range of mean effect sizes under the null hypothesis was also consistently larger than zero (95% CI calculated across the 1,000 iterations: 0.165–0.261), which covered our detected mean.

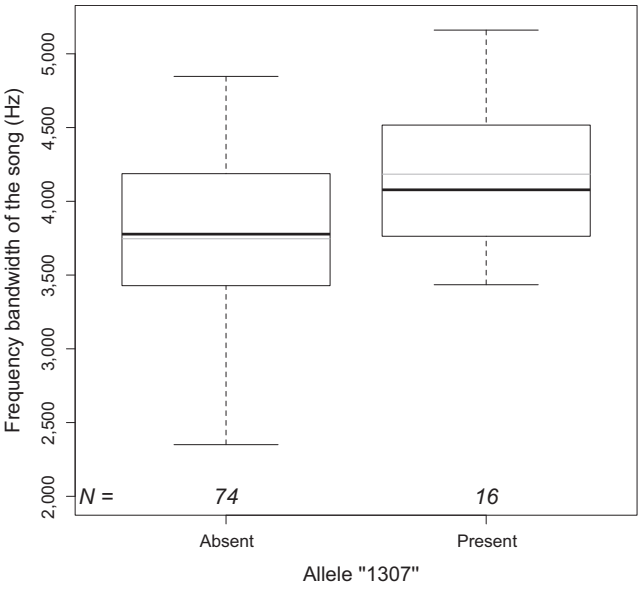

FIGURE 1 The relationship between the frequency bandwidth of the songs and the presence of the MHC class IIB second exon allele that is negatively associated with longevity (Supporting Information Figure S2) in the collared flycatcher. Boxplots show the extreme of the lower whisker, the lower hinge, the median, the upper hinge, and the extreme of the upper whisker, grey lines are for the group-specific means and numbers are the corresponding sample sizes

3.3 | Similarity in song and estimated genetic relationships based on MHC sequences

The matrix summarizing the pairwise distances among males based on the overlap in the syllable content of their songs was not significantly related to the matrix that includes the pairwise genetic distances relying on the amino acid sequences of the screened MHC region ($Z = 867.799$, $p = 0.767$; Supporting Information Figure S4).

4 | DISCUSSION

Here, as a pioneer effort, we investigated the potential relationship between features of song that can be used for female attraction and male–male competition and the characteristics of the MHC in a passerine bird. These investigations were motivated by the general prediction that these traits would be interrelated if songs have a function to signal genetically inherited parasite resistance or to enhance inbreeding avoidance. We performed a large number of comparisons and distinguished between the qualitative and quantitative aspects of both traits that can be involved in different mechanisms (Table 1). Despite these efforts, our findings were generally negative. We did not find statistical evidence for MHC allelic diversity (also reflecting heterozygosity) being related to either the estimates of song output and complexity or syllable composition. Furthermore, we detected no significant association between the similarity among males in their song content and their genetic similarity based on MHC. Finally, there was no indication that particular MHC alleles would be associated with the quantitative measures of songs. The only positive finding was that when we applied a biological rather than statistical inference, we detected a considerable relationship between the presence of an MHC allele that potentially mediates viability and the frequency bandwidth of the songs.

The nonindependent covariation of these traits is, in general, in accordance with the hypothesis stating that the courtship song of males bears information about MHC profile, and such information can mediate female choice alongside a model of sexual selection that emphasizes the role of a specific allele (Penn & Potts, 1999). In particular, the observation that males possessing an allele that may impair survival have songs that can be characterized by wider frequency ranges suggests that it may be beneficial for females to prefer males that sing at narrower bandwidths. Such males are unlikely to carry an MHC allele that makes individuals susceptible to parasitism and to have a genetic profile that incurs survival costs (see, e.g., Kloch, Baran, Buczek, Konarzewski, & Radwan, 2013; Loiseau et al., 2011; Lukasch et al., 2017). Singing at narrower frequency ranges may be more efficient in densely vegetated habitats, as it makes the signal less degradable or it allows the energy concentrated over small frequency bands for higher propagation (Boncoraglio & Saino, 2007). Alternatively, thinner frequency ranges can be coupled with song production with little within-song variability,

and such consistent singing performance may sometime confer advantage during female choice (Byers, 2007). Accordingly, only viable and healthy males (which lack the deleterious "1307" MHC allele) with superior quality may be able to bear the costs of the production of efficient and attractive signals.

Although we can only speculate about the mechanisms by which MHC alleles influence the frequency bandwidth of the song, it is very unlikely that the causal mechanism, if any, is as direct as it is for the olfactory system. Hence, for example, some by-products or derivatives of the MHC molecules may have modulator roles in the developing brain (Huh et al., 2000), through which the learning or crystallization of certain features of the song is also affected. Alternatively, both the immune system and the central nervous system may rely on the same precursor molecules at some stage of the early development, and this shared pathway during ontogeny may cause an apparent link between MHC expression and song production in adulthood. It is also plausible that there is no mechanistic bond between the two traits, but their covariation at the phenotypic level is the consequence of a noninvestigated property of individual quality. If males in good quality and/or condition are more likely to produce costly song traits at a narrow bandwidth and lack MHC alleles that make them less likely to survive (e.g., due to high susceptibility to parasitism), individual quality can act as a third variable that mediates a correlation between the respective song and MHC trait. Lastly, given that the genetic components of both the MHC (Ekblom et al., 2011) and the song control system in the brain (Lovell et al., 2008) involve hundreds of genes, this is inevitable that some of these become genetically linked. Such a linkage may be maintained for evolutionary timescales, if sexual selection favours a signalling mechanism that relies on this generic link. In any case, the potential role of the MHC molecules in the development of avian song deserves further investigations, for which the current findings can serve for motivation.

The large number of nonsignificant/weak relationships between MHC and song features that emerged in this study may arise by, at least, four different explanations. First, the chosen song variables may be poor indicators of male quality or may be irrelevant for female choice. However, this is a debatable explanation because many of the chosen acoustic characters in this and other species have been frequently shown to be repeatable, have a potential to indicate individual quality and can be connected to mating success or territory retention (reviews on other species: Byers & Kroodsma, 2009; Catchpole & Slater, 2008; Gil & Gahr, 2002; Searcy & Nowicki, 2000) (case studies in the collared flycatcher: Garamszegi et al., 2004, 2006, 2007, 2012). Second, the screened fraction of the MHC may be a poor indicator of parasite resistance or genetic relatedness. Given that we have not completely screened the MHC, it remains plausible that a more detailed analysis of the complex would yield stronger associations with song, because such a broader coverage might capture the regions that are relevant for the hypothesis under test (Sommer, 2005). However, there is evidence in this species showing that this fraction of MHC has pronounced sequence divergence depicting strong signals of selection, because it contains the antigen-binding sites with an excess of nonsynonymous substitutions (Zagalska-Neubauer et al., 2010), and because its functional diversity offers protection against avian malaria (Radwan et al., 2012). Third, the statistical approaches we have applied might suffer from lack of power. The large number of tests performed inherently increased the chances of type I errors when focusing on significance levels. Therefore, we applied procedures to account for multiple testing, but available control methods can do it at the cost of introducing type II errors only (Pike, 2011; Verhoeven, Simonsen, & McIntyre, 2005). Even though the FDR approach offers a reasonable compromise over the loss of power, it is remarkable that the most significant effect ($p = 0.0026$) became nonsignificant ($p = 0.251$) under the control by FDR. Considering the potentially indirect, if any, mechanistic link between song and MHC traits (as speculated above), we can assume that the investigated phenotypic relationships can be characterized with weak or immediate effect sizes at the best. Consequently, we cannot exclude the possibility that in our statistical framework, we failed to prove these effects with statistical significance. Noteworthy, when we relied on a biologically inferred interpretation, we could identify a meaningful association between a particular MHC allele and a song trait that could be characterized with an immediate effect size. Fourth, it might also be that there is truly no association between the investigated traits, and most features of song in the collared flycatcher do not evolve as reliable signals of individual MHC profiles. Or more broadly, birdsong is not shaped by MHC-mediated sexual selection. However, larger repertoire size was related to intermediate MHC diversity in the song sparrow (*Melospiza melodia*) suggesting that birdsong can bear signal functions in association with the MHC (Slade et al., 2017).

In birds, despite a list of evidence for MHC-dependent mating (e.g., Dunn et al., 2013; Griggio et al., 2011; von Schantz et al., 1997; Whittingham et al., 2015), candidate mechanisms for MHC signalling remain yet to be explored (Slade et al., 2016, 2017). This strikingly contrasts with our knowledge in other vertebrate taxa. Here, we proposed that birdsong, which depicts a tremendous variation, can evolve as a reliable indicator of individual MHC profiles and mediate female choice. For the moment, the mechanisms that generate a link between the signal and MHC traits are not as clear as for the chemical systems operating in many mammalian and fish species. Although we failed to deliver a strong evidence for attributes of songs in the collared flycatcher functioning as signals of MHC-mediated immunocompetence or kinship, our pioneer results and the established statistical framework may delineate new and interesting directions for future research. Furthermore, the large number of negative results in relation to acoustic traits may indicate that other modes of signalling should be considered, and olfactory perception in birds might be still an overlooked phenomenon.

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DATA ACCESSIBILITY

The data sets compiled for our analyses are deposited on Dryad (https://doi.org/10.5061/dryad.sd5hv94).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTION

L.Z.G. designed the study. L.Z.G., M.L., G.N., E.S., É.V., J.T., and S.Z. participated in the fieldwork, E.S. prepared samples, D.C. and M.Z.N. carried out the genetic analyses; S.Z. wrote the program for song analyses, L.Z.G., G.B., M.L., G.N., É.V., and S.Z. analyzed the song recordings; L.Z.G. performed the statistical analyses and wrote the paper. All authors provided comments on different versions of the manuscript.

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