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Extra-pair mating in a passerine bird with highly duplicated major histocompatibility complex class II: Preference for the golden mean

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Abstract

Genes of the major histocompatibility complex (MHC) are essential in vertebrate adaptive immunity, and they are highly diverse and duplicated in many lineages. While it is widely established that pathogen-mediated selection maintains MHC diversity through balancing selection, the role of mate choice in shaping MHC diversity is debated. Here, we investigate female mating preferences for MHC class II (MHCII) in the bluethroat (*Luscinia svecica*), a passerine bird with high levels of extra-pair paternity and extremely duplicated MHCII. We genotyped family samples with mixed brood paternity and categorized their MHCII alleles according to their functional properties in peptide binding. Our results strongly indicate that females select extra-pair males in a nonrandom, self-matching manner that provides offspring with an allelic repertoire size closer to the population mean, as compared to offspring sired by the social male. This is consistent with a compatible genes model for extra-pair mate choice where the optimal allelic diversity is intermediate, not maximal. This golden mean presumably reflects a trade-off between maximizing pathogen recognition benefits and minimizing autoimmunity costs. Our study exemplifies how mate choice can reduce the population variance in individual MHC diversity and exert strong stabilizing selection on the trait. It also supports the hypothesis that extra-pair mating is adaptive through altered genetic constitution in offspring.

KEYWORDS
bluethroat, compatibility, extra-pair mating, major histocompatibility complex, mate choice, optimal MHC

1 | INTRODUCTION

Genes of the major histocompatibility complex (MHC) constitute an important part of the adaptive immune system in vertebrates. They code for proteins that present intracellular (MHC class I; MHCI) and extracellular (MHC class II; MHCII) pathogen-derived antigens to T-cells and hence trigger an immune response against the specific pathogens (Janeway, Travers, Walport, & Shlomchik, 2001). The arms race between hosts and parasites contributes to the maintenance of extensive polymorphism through balancing pathogen-mediated selection (Spurgin & Richardson, 2010) via heterozygote overdominance (Doherty & Zinkernagel, 1975; Hughes & Nei, 1988, 1989), negative frequency-dependent selection (Bodmer, 1972; Slade & McCallum, 1992; Snell, 1968; Takahata & Nei, 1990) and fluctuating

selection (Hamilton & Zuk, 1982; Hedrick, 2002; Hedrick, Thomson, & Klitz, 1987). In addition to heterozygosity, gene duplications can expand intraindividual MHC repertoire and increase the number of pathogens that can be combatted (Nei, Gu, & Sitnikova, 1997).

The number of intraindividual MHC loci varies among species (e.g., Minias, Pikus, Whittingham, & Dunn, 2018). Duplications of MHC loci are presumably selected for when hosts are exposed to a broad array of pathogens (Westerdahl, Wittzell, & von Schantz, 2000). However, there are also costs of having a large number of MHC alleles; the risk of autoimmune diseases increases and the immune system could be less efficient due to negative selection of T-cells in the thymus (Lenz et al., 2015; Migalska, Sebastian, & Radwan, 2019; Nowak, Tarczy-Hornoch, & Austyn, 1992; Vidović & Matzinger, 1988, but see Borghans, Noest, & De Boer, 2003). This will lead to a trade-off in the number of intraindividual MHC alleles, and the optimal number could thus be expected to be intermediate rather than maximal (Kalbe et al., 2009; Wegner, Kalbe, Kurtz, Reusch, & Milinski, 2003; Woelfing, Traulsen, Milinski, & Boehm, 2009). It is conceivable that selection for an intermediate number of MHC alleles, i.e., the golden mean (Woelfing et al., 2009), could be especially pronounced in species possessing many MHC loci.

Whenever MHC affects fitness, females would be selected to choose a mate that will give rise to an optimal MHC diversity in the offspring (Milinski, 2006; Woelfing et al., 2009). Accordingly, genes of the MHC have been suggested to be candidate genes underlying female mate choice (Edwards & Hedrick, 1998; Penn & Potts, 1999; Yamazaki et al., 1976), but decades of studies of MHC and mate choice have rendered equivocal results (Kamiya, O'Dwyer, Westerdahl, Senior, & Nakagawa, 2014; Piertney & Oliver, 2006). If a female is capable of assessing her own MHC, she should choose a mate with a compatible genotype so that the diversity in the offspring will be optimal (Penn & Potts, 1999; Trivers, 1972). A preference for maximal MHC-dissimilar mates has been found in many vertebrates (e.g., Freeman-Gallant, Meguerdichian, Wheelwright, & Sollecito, 2003; Landry, Garant, Duchesne, & Bernatchez, 2001; Olsson et al., 2003; Strandh et al., 2012; Wedekind, Seebeck, Bettens, & Paepke, 1995; Yamazaki et al., 1976), while other studies have indicated choice for mates with intermediate dissimilarity (e.g., Baratti et al., 2012; Bonneaud, Chastel, Federici, Westerdahl, & Sorci, 2006; Eizaguirre, Yeates, Lenz, Kalbe, & Milinski, 2009; Forsberg, Dannewitz, Petersson, & Grahn, 2007), in line with the theoretical framework of an intermediate optimum. On the other hand, if females are not capable of self-referencing, they might choose mates with an optimal MHC diversity. Choice of the most MHC-diverse males has also been demonstrated in several taxa (e.g., fish [Reusch, Häberli, Aeschlimann, & Milinski, 2001], mammals [Ditchkoff, Lochmiller, Masters, Hoofer, & Bussche, 2001; Winternitz, Abbate, Huchard, Havlíček, & Garamszegi, 2017] and birds [Bonneaud et al., 2006; Dunn, Bollmer, Freeman-Gallant, & Whittingham, 2013; Richardson, Komdeur, Burke, & von Schantz, 2005; Whittingham, Freeman-Gallant, Taff, & Dunn, 2015]), while other studies have supported selection for males with an intermediate MHC diversity (e.g., Jäger et al., 2007; Slade, Watson, & MacDougall-Shackleton, 2017). In contrast, Dearborn et al. (2016) suggested that the benefits of MHC-based mate choice will be reduced in species with duplicated and diverged MHC loci, because diverse multilocus genotypes will then be inherited also under random mating. This could possibly explain the lack of MHC-based mate choice found by several studies (e.g., Paterson & Pemberton, 1997; Sepil et al., 2015; Westerdahl, 2004). Extending this argument further, MHC-based mate choice should be less pronounced in species with a high number of MHC loci.

Studying species that are socially monogamous but exhibit extra-pair paternity offers an opportunity to gain insights into the genetic basis of mate choice. While the social male might be chosen for his territory quality and parental abilities, the extra-pair male usually contributes only sperm and might be chosen for genetic benefits (Mays & Hill, 2004). Optimization of offspring MHC might confer such benefits, due to the importance of MHC in the defence against fast-evolving parasites (Milinski, 2006), but whether this holds true for species with extreme levels of MHC-diversity is not known.

Passerine birds are generally characterized by polygenic and polymorphic MHC (Westerdahl, 2007). In this study, we investigated the significance of MHC-based mate choice in a passerine species with highly duplicated MHC. The bluethroat (*Luscinia svecica*, Linnaeus, 1758) provides an excellent study system as it is among the bird species with the highest intraindividual MHCII diversity known to date (minimum 28 loci; Rekdal, Anmarkrud, Johnsen, & Lifjeld, 2018), and has an extensive extra-pair mating system (i.e., about 50% of the nests have extra-pair offspring; Johnsen & Lifjeld, 2003). Intriguingly, immunogenetic benefits of extra-pair copulations have indeed been suggested for this species: Johnsen, Andersen, Sunding, and Lifjeld (2000) and Fossøy, Johnsen, and Lifjeld (2008) found a higher immune response in extra-pair offspring than in both their maternal and paternal half-siblings. This suggests a preference for compatible genes in extra-pair mate choice in the bluethroat, which implies variable preferences that depend on the chooser's own genotype. In a previous study of this species, there were no correlations between male morphological traits within natural phenotypic variation and male success of extra-pair fertilisations (Johnsen, Lifjeld, Andersson, Örnborg, & Amundsen, 2001), which is consistent with a lack of directional selection on male secondary sexual traits through female choice of extra-pair males.

We based our analyses on bluethroat nests with known mixed paternities and identified genetic sires in order to be able to compare female choice of social males and extra-pair males. If females chose extra-pair males based on MHC compatibility, we predicted a difference in MHC diversity in the combined genotype of the female and her social mate and that of the female and her extra-pair male. Alternatively, if females based their choice on the male MHC genotypes alone, irrespective of their own genotype, we expected to find a difference between the intraindividual MHC diversity of the social male and the extra-pair male. Female MHC-based choice of extra-pair males should consequently lead to a difference in MHC diversity between within-pair and extra-pair offspring. If there was selection for maximized diversity, we expected a higher diversity

in extra-pair than in within-pair offspring. Conversely, if there was selection for intermediate diversity, we expected MHC diversity to be more concentrated around an intermediate optimum (i.e., lower variance; Forsberg et al., 2007; Lenz, Eizaguirre, Scharsack, Kalbe, & Milinski, 2009) in extra-pair offspring.

2 | MATERIALS AND METHODS

2.1 | Study population and data collection

The present study is based on part of the same data set as used in Johnsen et al. (2000) and Fossøy et al. (2008). Blood samples were collected from adult and nestling bluethroats from a wild population in Øvre Heimdalen, Øystre Slidre, Norway (61°25′N, 8°52′E) during the spring and summer of 1998 and 1999. Because about 50% of all bluethroat nests in the study population contain one or more extra-pair young (EPY), and about 26% of all offspring are EPY (Johnsen & Lifjeld, 2003), the genetic parentage of the chicks were decided based on microsatellites in the previously published studies (Fossøy et al., 2008; Johnsen et al., 2000). In this study, we included 279 individuals from 38 complete families in which both females, EPY, within-pair young (WPY), social males (WPM) and extra-pair males (EPM) were known and sampled. DNA was extracted using E-Z 96 Blood DNA Kit (Omega Bio-Tek Inc. [D1199-01]), following the manufacturer's protocol.

2.2 | Sequencing and allele calling of MHCIIβe2

All DNA samples were amplified in duplicates using the primers MHCIIFihy-E2CF and MHCIIFihy-E2CR (Canal, Alcaide, Anmarkrud, & Potti, 2010) and the sample indexing setup described by Fadrosh et al. (2014). Details regarding PCR conditions and thermal profile are presented elsewhere (Rekdal et al., 2018). The amplicons were sequenced on an Illumina MiSeq instrument using v3 chemistry. The workflow used to call the MHCIIβe2 alleles resembles closely the pipeline outlined by Rekdal et al. (2018), which is based upon the allele identification methodology published by Sommer, Courtiol, and Mazzoni (2013). The use of replicates and family information facilitated allele calling. Several measures were taken to avoid artefacts. In short, this included reducing the number of PCR cycles to 25 to minimize artefact formation (Lenz & Becker, 2008), as well as implementing a strict filtering scheme in the allele calling process. For details, see Appendix S1.

We successfully genotyped 24 females and 35 males sampled in 1998, 12 females and 21 males sampled in 1999 (one female and three males were recaptures from 1998), as well as 98 WPY and 86 EPY in total from the 38 nests from both years. Because two female samples failed, we had only 36 complete trios (female, social male and extra-pair male) left for analysis, but 38 male duos (social and extra-pair male). Five offspring samples also failed during sequencing, leaving no WPY genotyped for one nest and no EPY genotyped for another. Thus, only 36 nests contained both WPY and EPY and were used for paired comparisons of the two groups. Several males sired both WPY and EPY in this data set. Every EPM also sired WPY in their own nest, although not all of these nests were included in this study. All pairs (i.e., combinations of male and female identity) were unique.

2.3 | Establishing genotypes for PSS alleles and supertypes

In order to consider the functional aspects of the MHC alleles, we employed the program CodeML in the package PAML (Yang, 2007) to identify sites under positive selection (positively selected sites; PSS). These are sites that probably are under pathogen-mediated selection, and thus presumably are important in antigen binding and hence the function of MHCII (Hughes & Nei, 1989; Sepil, Moghadam, Huchard, & Sheldon, 2012; Yang & Swanson, 2002). CodeML uses a codon substitution model on the sequence phylogeny to accomplish a likelihood ratio test (LRT), comparing a model with no positive selection (M7: dN/dS < 1) with a model that allow positive selection at amino acid sites (M8: dN/dS > 1). As the M8 model fitted the data significantly better than M7 (see Appendix S2), a Bayes Empirical Bayes-procedure (BEB) was used to identify sites under positive selection (p > 95%) through a maximum likelihood framework. Of the 12 residues identified as PSS by CodeML, eight have also been described as antigen binding residues in bluethroats (Gohli et al., 2013, which again is based on the PBR of human MHCII by Tong et al., 2006) and in other passerines (Balakrishnan et al., 2010). These eight residues were thus selected as the basis for PSS sequences in this study: 4, 6, 8, 23, 25, 52, 55 and 66.

The unique PSS sequences were further subdivided into supertypes based on the physiochemical properties of the amino acid residues through z-descriptors (Sandberg, Eriksson, Jonsson, Sjöström, & Wold, 1998), aiming to group the PSS sequences with similar antigen binding properties (Doytchinova & Flower, 2005; Sepil et al., 2012). The R package adegenet (Jombart, 2008) was employed to infer clusters (i.e., supertypes) by k-means clustering (Doytchinova & Flower, 2005). There were 20 supertypes inferred from the PSS sequences. Details are given in Appendix S2. The number of unique PSS sequences per supertype ranged from four (cluster 5) to 25 (cluster 8), with an average of 15.6.

We designated genotypes for PSS alleles and supertypes for each individual based on their nucleotide genotype (Appendices S2 and S5), and used the PSS and supertype genotypes in all downstream analyses. We also obtained the number and identity of unique PSS alleles and supertypes within a pair, for all established pairs (social pairs [henceforth WPM-F], and extra-pair partners [EPM-F]).

2.4 | Statistical analyses

2.4.1 | Male diversity

To test for female choice of EPM for maximum male diversity, we computed the number of unique PSS alleles, sum of the amino acid distance between all pairs of unique PSS alleles, average amino acid

distance between all pairs of unique PSS alleles and the number of supertypes for the individual males. Paired *t* tests were run to evaluate if there were any differences in the mean values of the parameters for WPM and EPM.

If females choose males with an intermediate number of alleles as EPM, we expect that the observed values will be more concentrated around an optimum in EPM than in WPM. We thus tested if the variances in the number of unique PSS alleles and supertypes differed between WPM and EPM using Levene's test (Brown-Forsythe type; Brown & Forsythe, 1974) for equality of variances, in the R package car (Fox & Weisberg, 2018). The intraindividual number of PSS alleles was further examined by paired *t* test, testing the distance to yearly population mean for WPM and EPM.

We built linear models to test if there were any significant correlations between the number of PSS alleles in the females and the males (WPM/EPM), in all observed pairs.

2.4.2 | Compatibility

If the females choose EPM based on her own genotype in order to maximize the MHCII diversity in her offspring, only the unique, nonshared alleles of the pair found exclusively within the males would be relevant for her choice. For every observed pair, we thus established these parameters: the number of nonshared PSS alleles only found within the male, sum of the amino acid distance between all pairs of nonshared PSS alleles only found within the male, average amino acid distance between all pairs of nonshared PSS alleles only found within the male and the number of nonshared supertypes only found within the male. Paired *t* tests were conducted in order to test if there were any differences in the abovementioned parameters between WPM-F and EPM-F within nests. We also employed Welch's unequal variances *t* test to further explore the differences between WPM-F and EPM-F in the total number of unique PSS alleles found within a pair.

As for WPM and EPM, we employed Levene's test to test the equality of variances in the observed number of PSS alleles and supertypes in WPM-F and EPM-F. If females choose males who render an intermediate number of alleles in the pair as EPMs, we correspondingly expect a smaller variance in EPM-F than in WPM-F. The difference between WPM-F and EPM-F in their distance to yearly population mean number of PSS alleles in the pair was also tested using a paired *t* test as well as Welch's unequal variances *t* test. Further, we ran simulations to test whether the observed numbers of PSS alleles within mating pairs differed from a random model. For each run in the simulations, we paired each female with a random male sampled in the same year, calculated the number of unique PSS alleles for the pair and listed its deviation (absolute value) from the overall population mean. We then calculated and recorded the mean deviation across all 36 females in our sample. This procedure was iterated 10,000 times, which yielded a distribution of 10,000 means, to which the observed means for social pairs and extra-pair partners could be compared.

2.4.3 | Offspring

Mate choice can also be tracked in the genotypes of offspring. If there is selection for females to choose EPM that will maximize MHCII diversity in the offspring, we expect EPY to have a higher number of PSS alleles or supertypes than WPY. We thus performed paired *t* tests on the number of PSS alleles and the number of supertypes in WPY and EPY, paired within nests. We also used the R package lmerTest (Kuznetsova, Brockhoff, & Christensen, 2017) to build a linear mixed model of the correlation between the number of PSS alleles (response) and the status of the offspring (WPY/EPY; fixed effect), with dam and sire identity as random factors with random intercepts. We further established a corresponding linear mixed model to test if the distance to the population average number of PSS alleles was different in WPY and EPY. For the mixed models, we square root transformed the response variables (i.e., the number of PSS alleles and the distance to population average in absolute numbers of PSS alleles) to attain normality. We also performed a paired *t* test to test for a difference in the distance to the population average number of PSS alleles between WPY and EPY within nests. Similarly as for the adults, we carried out tests for equality of variances in the intraindividual number of PSS alleles (*F*-ratio test; see below) and supertypes (Levene's test) between WPY and EPY. Given the results we obtained from the adults, we had an expectation of EPY being closer to the population mean in the intraindividual number of PSS alleles than WPY. We thus applied one-sided tests testing this hypothesis (valid for two tests: the paired *t* test testing if EPY had a smaller distance to the population average number of PSS alleles than WPY, and a one-sided F-ratio test testing if EPY had a smaller variance in the number of PSS alleles than WPY). We square root transformed the intraindividual numbers of PSS alleles for the latter test, as it is highly sensitive to deviation from normality. For the variance tests and paired *t* tests in offspring, one WPY and one EPY were chosen randomly from each nest and used in the analyses, in order to avoid pseudoreplication. The analyses were repeated 10,000 times, and the resulting mean *t*- and *F*-values were used for calculation of *p*-values.

2.4.4 | For all analyses

Only the male with the highest number of offspring in the respective nest was included when there were two EPMs genotyped for one nest (relevant in four nests in this data set). All the statistical analyses were conducted in R (R Core Team, 2016, see Rekdal, Anmarkrud, Lifjeld, & Johnsen, 2019 for scripts and input data), while the amino acid distances were calculated by MEGA7 (Kumar, Stecher, & Tamura, 2016). The distance to the population average number of PSS alleles was square root transformed to endeavor normality in all tests using this variable. Visual inspection of QQ-plots and Shapiro–Wilk tests revealed normality for all tests (data not shown), with exception of slight deviation from normality in the Welch's *t* test between WPM-F and EPM-F in their distance

TABLE 1 The number of unique MHC class II variants at the different sequence levels, found across all individuals (adults and offspring) and within individuals (given as mean values and range)

Sequence level	Number of unique variants across all individuals	Alleles per individual	Alleles per adult	Alleles per offspring
Nucleotide	1,176	35.2 (16–58)	37.7 (16–58)	34.1 (19–52)
Translated	890	31.3 (16–47)	33.4 (16–47)	30.3 (18–45)
PSS	311	18.6 (7–32)	19.8 (11–32)	18.0 (7–32)
Supertypes	20	12.0 (5–18)	12.4 (8–18)	11.7 (5–18)

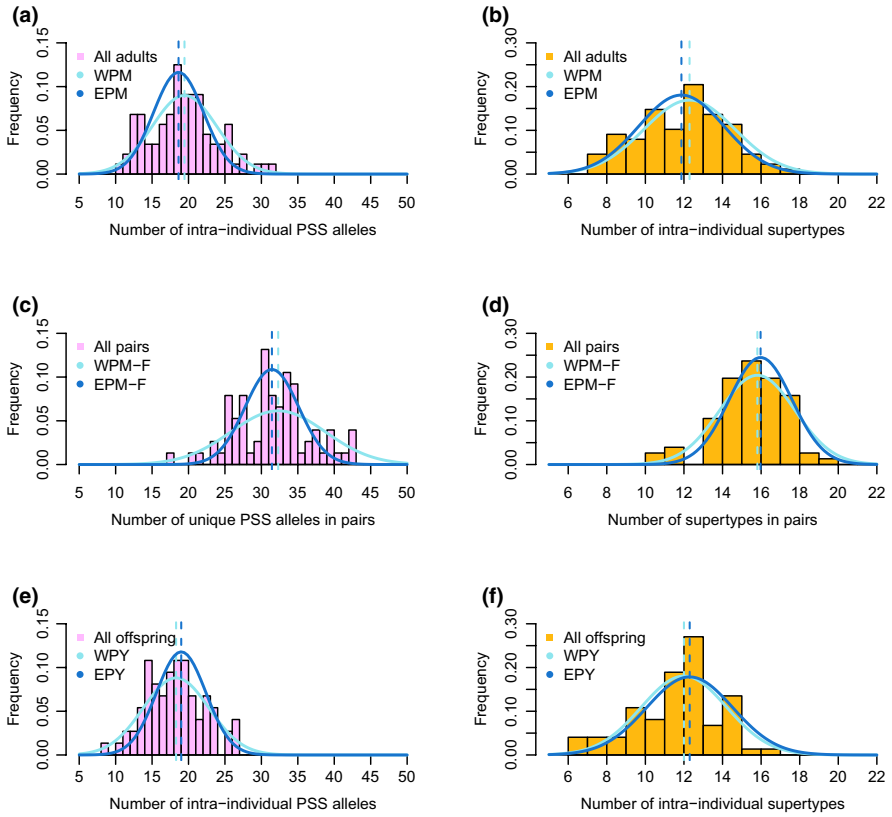

FIGURE 1 Frequency plots of the number of unique MHC class II PSS alleles (positively selected sites; left) and supertypes (right) found within all individual adults (top panel [a, b]), all observed partners (middle panel [c, d]) and offspring (bottom panel [e, f]). The observed values for social and extra-pair males (WPM/EPM [a, b]), social and extra-pair partners (WPM-F/EPM-F [c, d]) and within-pair and extra-pair young (WPY/EPY [e, f]) are visualized as normalized curves in light (WPM, WPM-F, WPY) and dark (EPM, EPM-F, EPY) blue. The mean of the normalized curves are given as dashed lines and coloured correspondingly

to yearly population mean number of PSS alleles in the pair. We approximated the optimal intermediate number of PSS alleles as the mean intraindividual number of alleles across all adults sampled within a year (1998:19.9 PSS alleles per individual, 1999:19.8 PSS alleles per individual), and the mean number of unique alleles within a pair over all possible pairs within a year (1998:32.0 PSS alleles per pair, 1999:32.7 PSS alleles per pair). This agrees with work on sticklebacks (*Gasterosteus aculeatus*) showing that the estimated optimum number of alleles is close to population average (Aeschlimann, Häberli, Reusch, Boehm, & Milinski, 2003). All significant tests remained significant after controlling for multiple testing using false discovery rate (Benjamini & Hochberg, 1995; $Q = 0.1$).

3 | RESULTS

3.1 | High number of MHCII alleles within individuals

The 1,176 nucleotide MHCII sequences translated into 890 unique amino acid sequences. When considering only the eight amino acid residues recognized as PSS, the sequences grouped into 311 unique PSS alleles, which further were divided into 20 supertypes (see Table 1 and Appendix S3). Across all adults sampled both years, the mean intraindividual number of nucleotide alleles, PSS alleles and supertypes were 37.7 ($SD = 8.28$), 19.8 ($SD = 4.60$) and 12.4 ($SD = 2.32$), respectively. The frequencies of each number of unique PSS alleles

and supertypes within individual adults, pairs and offspring are visualized in Figure 1.

3.2 | Do females choose EPM based on male MHCII diversity?

There were no indications of females choosing males with maximized diversity as EPM, as there were no significant differences in the mean parameter values between WPM and EPM (paired *t* tests; see Appendix S4). EPMs did also not have a number of PSS alleles that was closer to the population mean than that of WPMs (paired *t* tests; $t_{37} = 1.34$, $p = .19$). Correspondingly, the variances in the individual number of PSS alleles and supertypes within WPM and EPM were not significantly different (Levene's test for equality of variances; $F_{1,74} = 2.93$, $p = .091$ [PSS alleles; Figure 1a] and $F_{1,74} = 0.026$, $p = .87$ [supertypes; Figure 1b]).

The numbers of PSS alleles found within the females and EPMs were negatively correlated (linear models; $R^2_{adj} = 0.16$, $F_{1,34} = 7.51$, $p = .0097$), but were uncorrelated between females and WPMs ($R^2_{adj} = 0.012$, $F_{1,34} = 1.42$, $p = .24$). The corresponding regression slopes for EPM and WPM were significantly different ($F_{1,68} = 6.28$, $p = .015$, see Figure 2). In other words, this suggests that females with few PSS alleles tended to choose EPM with many PSS alleles and vice versa, but this pattern was not found for female choice of WPM.

3.3 | Do females choose EPM based on compatibility at MHCII?

Females did not consistently choose a more dissimilar extra-pair male than their social male (paired *t* tests; see Appendix S4). There was further no significant difference in the average number of unique PSS alleles in the pair between WPM-F pairs and EPM-F pairs (Welch's *t* test; $t_{55.3} = 0.69$, $p = .49$, see Figure 3a). However, the number of unique PSS alleles in EPM-F pairs showed less variance than in WPM-F pairs (Levene's test; $F_{1,70} = 9.54$, $p = .0029$; Figure 1c), but not so for the number of supertypes ($F_{1,70} = 0.99$, $p = .32$; Figure 1d). EPM-F pairs were also significantly closer to the population mean number of unique PSS alleles within pairs than WPM-F pairs (paired *t* test; $t_{35} = 3.05$, $p = .0043$, effect size = 0.62, Welch's *t* test; $t_{64.4} = 2.65$, $p = .010$, see Figure 3b).

Assuming random mating, we calculated 10,000 averages of the number of unique PSS alleles within pairs across all females, and their deviations from the population mean. Figure 4 shows the distribution of these deviations. We used this distribution to test if the observed average number of unique alleles in EPM-F and WPM-F deviated significantly from a random model. We found that only 33 of the 10,000 simulated means had a lower value than the observed EPM-F (exact test: $p = .0068$, Figure 4), while the observed mean value for WPM-F was ranked as the 9091st observation when sorted ascendingly (exact test: $p = .18$).

3.4 | Offspring

In line with the results from the adults, we found no support for female choice of EPM for maximized diversity in the offspring data: there were no differences in mean number of alleles between WPY and EPY, neither in the paired *t* tests (PSS alleles: $t_{35} = 0.12$, $p = .91$, supertypes: $t_{35} = -0.45$, $p = .66$) nor in the linear mixed model for PSS alleles ($t_{54.7} = -0.26$, $p = .80$, see Figure 3c). Furthermore, in line with the above results for mate choice, we found that EPY had a number of PSS alleles that was significantly closer to the population average than that of WPY (linear mixed model: estimate = 0.31, $t_{49.6} = 2.68$, $p = .0099$, see Figure 3d, paired *t* test: $t_{35} = -2.01$, $p = .026$). Similarly, there was a tendency for EPY to have a smaller variance in the individual number of PSS alleles than WPY (F ratio test; $F_{36,35} = 1.67$, $p = .066$, see Figure 1e), but not in the number of supertypes (Levene's test; $F_{1,71} = 0.39$, $p = .54$, see Figure 1f).

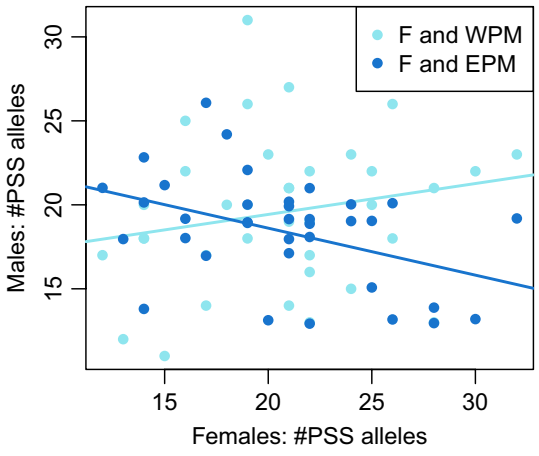

FIGURE 2 The number of unique MHC class II PSS (positively selected sites) alleles genotyped within each individual bluethroat in all observed pairs, divided in females and their social males (F and WPM; light blue), and females and their extra-pair males (F and EPM; dark blue)

4 | DISCUSSION

We have shown here that extra-pair mating in the bluethroat was nonrandom with respect to MHCII alleles. The number of unique, functional MHCII alleles among extra-pair parents was consistently closer to the population mean, with a significantly reduced variance, than that expected from a random model of pair combinations or that observed among social parents. Consequently, we found that extra-pair offspring received an allelic repertoire closer to the population mean than was the case for within-pair offspring. Our study therefore supports the hypothesis that females engage in extra-pair mating for genetic benefits and suggests that MHCII genes play a significant role in their mating preferences. Our results imply that females may be able to differentiate among alleles according to sequence variation at positively selected nucleotides in the peptide-binding region of the molecule, and not according to other physical properties assumed in the categorization of alleles into supertypes.

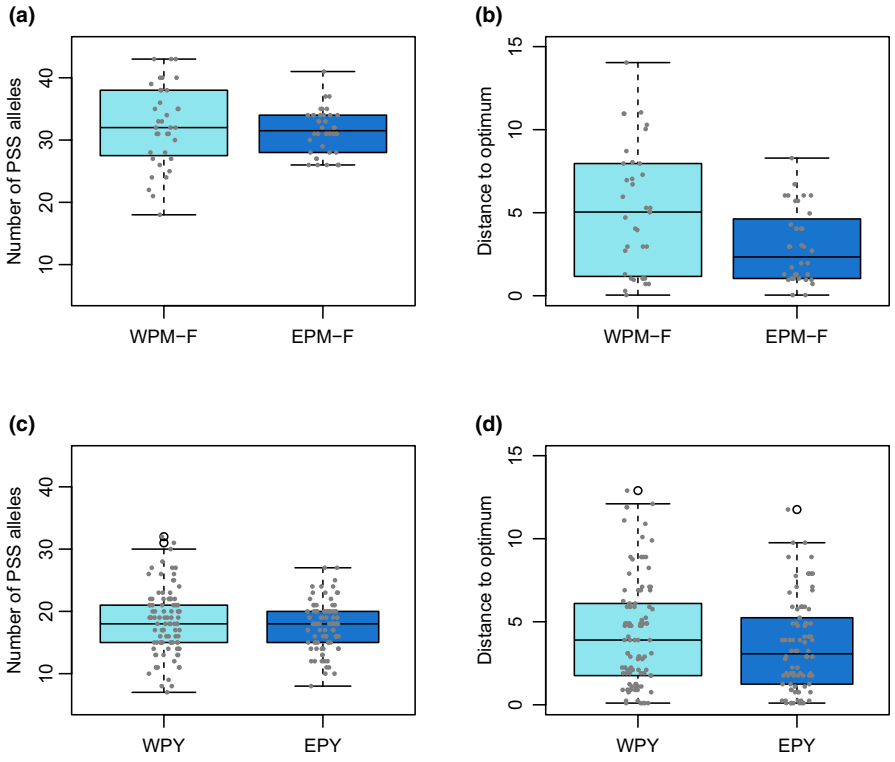

FIGURE 3 Boxplots of the observed values (grey, jittered dots) of the number of unique MHC class II PSS alleles (positively selected sites; a, c) and the absolute distance to the optimum (given in number of unique PSS alleles [nontransformed data]; b, d) within each pair in the data set, divided in social pairs and extra-pair partners (WPM-F/EPM-F [a, b]) and within each offspring, divided in within-pair young and extra-pair young (WPY/EPY [c, d])

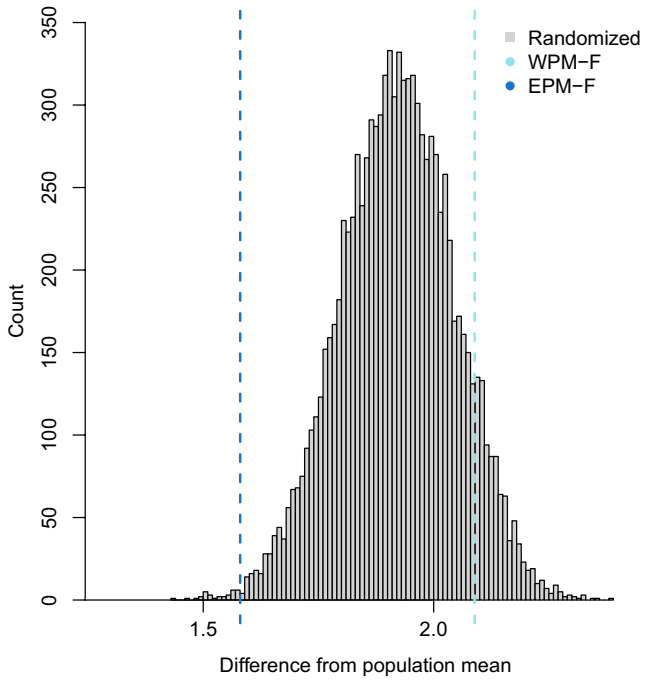

FIGURE 4 Histogram showing 10,000 simulated mean values of the distance to the yearly population mean (given in number of unique MHC class II PSS [positively selected sites] alleles within a pair, square root transformed), allowing each bluethroat female to mate with a random male within the data set. The observed mean values for social pairs (WPM-F) and extra-pair partners (EPM-F) are given as coloured, dashed lines (light blue and dark blue, respectively)

4.1 | MHC-based mate choice realized through extra-pair copulations

Dearborn et al. (2016) suggested that MHC-based mate choice may be superfluous in species with duplicated and diverged MHC loci, since offspring will inherit a diverse MHC genotype irrespective of mate choice. In contrast, we found substantial support for MHCII-based mate choice in a species possessing extensive MHCII duplications. Although we did not find any indications of disassortative mating with respect to MHCII, our results suggest that females choose extra-pair males that will render an intermediate number of functional MHCII alleles in the pair, leading to an intermediate, presumably optimal MHC diversity in extra-pair offspring.

A difference between within-pair and extra-pair units is expected if females choose social males for other than pure genetic benefits, while the extra-pair males might be chosen on the basis of their genes, as they probably only contribute sperm (Johnsen et al., 2001; Trivers, 1972). In the context of MHC, few studies on passerines have tested such differences, and among those which have, the results are mixed. Most studies did not find any support for increased compatibility between the MHC genotypes of the female and extra-pair male, compared to the female and the social male (Bollmer, Dunn, Freeman-Gallant, & Whittingham, 2012; Promerová et al., 2011; Richardson et al., 2005). Yet, Winternitz et al. (2015) found that in the scarlet rosefinch (*Carpodacus erythrinus*), the variance in intraindividual number of MHC alleles was lower in extra-pair offspring than within-pair offspring, which is consistent with

our results. They did, however, not report any differences in MHC compatibility between social and extra-pair partners.

Other studies have found that paternity loss from social to extra-pair males could be negatively associated with MHC diversity, based on either the social male's MHC diversity (Promerová et al., 2011; Richardson et al., 2005) or MHC similarity in the social pair (Freeman-Gallant et al., 2003) – a pattern also found in a primate with high levels of extra-pair paternity (Schwensow, Fietz, Dausmann, & Sommer, 2008). While we have only included pairs with confirmed cuckoldry in this study, and hence do not know the MHCII compatibility in pairs with pure WPY broods, we cannot test predictions concerning overall paternity loss in relation to partner compatibility in MHCII genotypes with our data.

4.2 | Intermediate, not maximized MHCII diversity

Our results point to selection for an intermediate optimum number of MHCII alleles, rather than maximized MHCII diversity in the bluethroat. This is in line with the theoretical framework of a trade-off between recognizing a broad array of pathogens, and increased depletion of circulating T-cells following negative selection in the thymus and risk of autoimmune diseases with increased number of MHC alleles (e.g., Gough & Simmonds, 2007; Nowak et al., 1992; Woelfing et al., 2009). What level of intraindividual MHC diversity that constitutes the optimum might vary among species due to ecological differences, e.g., according to the pathogen load experienced (Minias et al., 2018; O'Connor, Cornwallis, Hasselquist, Nilsson, & Westerdahl, 2018; Westerdahl et al., 2000). This implies that the more pathogens a species is exposed to, the stronger the selective force for increased diversity will be, driving the optimum towards a higher diversity. Because the bluethroat is migratory, insectivorous and fairly promiscuous, it probably encounters a multitude of pathogens. While this could explain the large number of MHCII loci in the species (Anmarkrud, Johnsen, Bachmann, & Lifjeld, 2010; Gohli et al., 2013; Rekdal et al., 2018), it is, however, important to emphasize that we did not find support for selection for maximized diversity, but rather indications of stabilizing selection for a relatively high intermediate number of MHCII alleles. Still, stabilizing selection can lead to an increase in individual allelic diversity over evolutionary time, through a moving intermediate optimum.

Fossøy et al. (2008) compared microsatellite multilocus heterozygosity between within-pair and extra-pair units in a bluethroat data set including the data used in this study. They found that females were less genetically similar to the extra-pair male than the within-pair male, which presumably explained their results of extra-pair young being more heterozygous than their maternal within-pair half-siblings. Our results do, however, exhibit a different pattern; instead of increased MHCII diversity, we found that extra-pair partners and extra-pair young had a number of unique PSS alleles that were more concentrated around the population mean than their within-pair counterpart. This deviates from what we would expect based on microsatellites from Fossøy et al. (2008), and indicates that what we observed on MHCII was not due to genome-wide effects.

We restricted our analyses to MHCII. The bluethroat has relatively few MHCI loci (i.e., four; O'Connor, Strandh, Hasselquist, Nilsson, & Westerdahl, 2016; Rekdal et al., 2018), which might be due to less exposure to intra- than extracellular pathogens (Minias et al., 2018) or some compensatory immunological mechanism (e.g., Gangoso et al., 2012; Star et al., 2011). Recent studies have identified a link between MHCII composition and individual odor in birds, possibly mediated through microbial communities and uropygial gland secretions (Leclaire et al., 2019, 2014; Leclaire, Strandh, Mardon, Westerdahl, & Bonadonna, 2017; Slade et al., 2016; Strandh et al., 2012). As there is growing evidence that birds are able to use olfaction in MHC-based mate choice, also in a self-referencing manner (reviewed by Caro, Balthazart, & Bonadonna, 2015), MHCII is a prominent candidate for such a mate choice mechanism.

4.3 | Compatibility, not male diversity

Unlike selection for maximized or intermediate diversity in the male, in which the same males will be the best choice for all females, selection for compatibility implies that the best choice a female can make is dependent on her own genotype (Brown, 1997). One suggested approach for MHC-based mate choice is allele counting, in which females assess the number of MHC alleles in males, and choose mates accordingly (Aeschlimann et al., 2003; Reusch et al., 2001). A trend of mating-up preference by allele counting was supported by Griggio, Biard, Penn, and Hoi (2011), who found that female house sparrows (*Passer domesticus*) with a low number of MHC alleles preferred high diversity males. We found the same tendency in this study; females with a low number of MHCII alleles had males with a high number of alleles as extra-pair males, and those with many alleles had males with fewer alleles as extra-pair males (Figure 2).

Importantly, all our tests on the adult bluethroats rendered significant results only when considering MHCII diversity in the pairs combined, and not in the male alone. These results are consistent with the compatibility framework (Brown, 1997; Trivers, 1972), where females choose mates based on their own genotype in order to produce offspring with an optimal MHC diversity (Penn & Potts, 1999). Indeed, Johnsen et al. (2000) and Fossøy et al. (2008) demonstrated an increased immunocompentence in extra-pair offspring as compared to both their maternal and paternal half-siblings. Taken together, these studies offer extensive support for the compatibility hypothesis, realized through extra-pair mating in the bluethroat.

4.4 | Positive selected sites, not supertypes

The significant results in this study originated from analyses on PSS alleles, and not supertypes. The rationale for further grouping the PSS sequences into supertypes was to focus on a possible higher unit

of selection, due to the overlap in binding repertoires by different MHC alleles (Matsumura, Fremont, Peterson, & Wilson, 1992; Sepil et al., 2012; Sette et al., 1989; Sette & Sidney, 1998; Trachtenberg et al., 2003). However, our results do not indicate any female discrimination of MHCII alleles at the level of supertypes. One conceivable explanation for the disparate results could be due to information loss in the grouping of PSS alleles into supertypes. Another possibility could be spurious inference of irrelevant properties of the supertypes. Supertypes might be functionally important in pathogen recognition, but it is possible that females can only discriminated MHCII alleles from information encoded in their nucleotide sequences.

5 | CONCLUSION

In conclusion, this study provides substantial support for extra-pair mating preferences associated with MHCII diversity in a passerine species with highly polymorphic and duplicated MHCII. The results are in agreement with a preference for a golden mean where an intermediate number of alleles in the individual is optimal, given an assumed trade-off between maximizing the range of pathogens that can be combatted and minimizing autoimmunity costs associated with too many alleles. We note, however, that we currently lack fitness data to verify that individuals with intermediate level of PSS alleles have higher survival than those at the more extreme ends of the allele number distribution. Our study provides additional empirical support for the hypothesis that females engage in extra-pair mating nonrandomly. It further suggests that this behaviour is associated with the genetic constitution of the immune system and the survival prospects of offspring under strong pathogen-mediated selection pressures.

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AUTHOR CONTRIBUTIONS

S.L.R., J.A.A., J.T.L., and A.J. designed the study. J.A.A. carried out the laboratory work. S.L.R. analyzed the data and drafted the manuscript which was revised by all authors.

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DATA AVAILABILITY STATEMENT

MHCIIβe2 nucleotide alleles are available in GenBank (accession numbers MN332585–MN333760). Raw sequence data are available through the NCBI Sequence Read Archive (BioProject accession number PRJNA560776). A bash script with the codes for making the jMHC input file from the raw data, as well as a R script for the statistical analyses and associated input files are deposited in Dryad (https://doi.org/10.5061/dryad.93tf68k). Individual genotypes, number of PSS alleles within pairs, nest affiliations, grouping of nucleotides into PSS alleles and supertypes, as well as nucleotide and PSS sequences can be extracted from Appendix S5. Barcodes and primer sequences are given in Appendix S6. The corresponding GenBank accession number to each nucleotide sequence allele used in this study is given in Appendix S7. Information on sampling and identification for each individual in the data set can be retrieved via the online Collection Explorer (http://nhmo-birds.collectionexplorer.org/accession.aspx), using the accession numbers found in Appendix S5.

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SUPPORTING INFORMATION

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

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