**ORIGINAL ARTICLE**

# Do female bluethroats without extra-pair offspring have more MHC-compatible social mates?

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## Abstract

Genes of the major histocompatibility complex (MHC) are crucial for adaptive immunity in jawed vertebrates, and theory predicts that there should be mate choice for optimizing MHC constitution in the offspring. In a previous study, we demonstrated a non-random female choice of extra-pair males in the bluethroat (*Luscinia svecica*), yielding offspring that was closer to an intermediate MHC class II (MHCII) allele count than their within-pair halfsiblings. The present study tests whether social pairs with only within-pair young (WPY) in their brood, in the same study population, had a combined MHC-constitution closer to a presumed intermediate optimum, than social pairs with extra-pair young (EPY), with a corresponding pattern in their offspring. As expected, we found that WPY from pure WPY-broods were more MHC-optimal than WPY from mixed broods, but only in broods of young (second year) males. Correspondingly, there was a tendency for social pairs with only WPY in their brood to be more MHC-compatible than social pairs with EPY in their brood, when the male was young. Older bluethroat males have considerably larger testes than young males, and their higher sperm competitiveness could help them secure paternity in their own brood, also when they are not MHC-compatible. In other words, in the sexual conflict over paternity, females may be more likely to realise their preference for a MHC-compatible mate when paired to a young male. As a possible fitness indicator, immune responsiveness to an injected antigen (PHA) was elevated for offspring closer to "the golden mean" in MHCII allele count.

## Significance statement

This study contributes to our understanding of MHC-based mate choice in extra-pair mating systems, by showing that female bluethroats (*Luscinia svecica*) with an MHCII-compatible social mate tend to have no extra-pair young in their brood, but only when the social male is young. This elucidates a possible sexual conflict, in which older social males are able to override female preferences and prevent other males from gaining paternity in their brood through higher sperm production. Studying systems in which extra-pair paternity occurs offers an insight into the genetic benefits of mate choice, as extra-pair males, in contrast to social males, generally contribute only sperm. Further, the strict and thorough genotyping scheme applied in this study enabled us to demonstrate a preference for "the golden mean" in MHC-diversity in a species with one of the highest MHC class II-diversity known to date.

**Keywords** Major histocompatibility complex · MHC · Mate choice · Sexual conflict · Extra-pair paternity · Bluethroat

## Introduction

Every organism needs to fight the continuous threat from pathogens, and there is therefore strong selection for optimising the ability of offspring to resist infectious diseases.

Given that potential mates vary in immunologically important genes, and that it is possible to assess these differences, the individuals are predicted to choose mates that render offspring with an optimal constitution of such genes (Milinski 2006). Genes of the major histocompatibility complex (MHC) are highly variable and essential components of adaptive immunity in jawed vertebrates and are therefore candidate genes for such pathogen-mediated selection via mate choice.

MHC genes code for transmembrane glycoproteins that present pathogen-derived antigens to T cells, thereby initiating immune responses against the specific pathogen

(Janeway et al. 2001). MHC class I (MHCI) proteins are found on all nucleated cells and present antigens from intracellular pathogens to cytotoxic $CD8^+$ T cells (Zinkernagel and Doherty 1974), while MHC class II (MHCII) proteins are only found on specialized antigen presenting cells like B cells, macrophages, and dendritic cells and present antigens from extracellular pathogens to $CD4^+$ T cells (Janeway et al. 2001). The number of unique MHC alleles found within an individual varies greatly among species, especially within Aves (O'Connor et al. 2019). While galliforms and birds of prey have only a few loci (Kaufman et al. 1999; Eimes et al. 2013; Minias et al. 2018), passerines exhibit multiple loci that have evolved through extensive gene duplication (Nei et al. 1997; Minias et al. 2018; Westerdahl et al. 2022). For instance, 33 MHCI loci have been reported in the sedge warbler (*Acrocephalus schoenobaenus*; Biedrzycka et al. 2017), and the expression of multiple MHCI loci was demonstrated in Eurasian siskins (*Spinus spinus*) by Drews and Westerdahl (2019). Bluethroats (*Luscinia svecica*) have a hypervariable MHCII (Rekdal et al. 2018), with up to 58 different alleles scored in an individual (Rekdal et al. 2019a). The MHC allele count varies among individuals within a species (Reusch et al. 2001; Woelfing et al. 2009; O'Connor et al. 2016), and in outbred species, the theory predicts that the optimal intra-individual allele count is at an intermediate rather than a maximized level (Nowak et al. 1992; Milinski 2006; Woelfing et al. 2009). This is based on a postulated trade-off between the ability to recognize and fight off more pathogens, and the risk of autoimmune diseases and fewer circulating T cells due to negative selection in the thymus (Doherty and Zinkernagel 1975; Vidović and Matzinger 1988; Lenz et al. 2015; Migalska et al. 2019). A higher fitness at an intermediate MHC allele count has been demonstrated in several taxa (Wegner et al. 2003; Madsen and Ujvari 2006; Kalbe et al. 2009; Kloch et al. 2010, but see Sepil et al. 2013).

Based on this theory, females should choose mates with a compatible MHC allelic repertoire, resulting in offspring with an intermediate MHC allele count. Previous studies on MHC-based mate choice have given mixed results (Kamiya et al. 2014). While some studies report choice for males with specific alleles, likely protecting against prevalent pathogens (e.g., Ekblom et al. 2004; Eizaguirre et al. 2009), others have found evidence for mate choice based on intermediate (e.g., Forsberg et al. 2007; Baratti et al. 2012) or maximized dissimilarity (e.g., Yamazaki et al. 1976; Strandh et al. 2012). Aeschlimann et al. (2003) found that female three-spined sticklebacks (*Gasterosteus aculeatus*) preferred males that resulted in offspring with an allele count close to the population mean, which also is close to the MHC diversity with lowest pathogen load (Wegner et al. 2003). Thus, mating preferences seem to cause stabilizing selection for an optimal allele count at the population mean in that system.

The bluethroat is a socially monogamous passerine with high levels of extra-pair paternity (Questiau et al. 1999; Johnsen and Lifjeld 2003). The male age is an important determinant of paternity patterns in this species, as older males have larger testes and thus higher sperm production rates than young males (Laskemoen et al. 2008). They are also more successful in obtaining extra-pair fertilizations than young males, and they maintain the same level of paternity in their broods despite spending less time with the female (Johnsen et al. 2001, 2003). In an earlier study, we found that extra-pair offspring had higher immune responsiveness than their within-pair half siblings (Johnsen et al. 2000). Immune responsiveness was estimated by the phytohaemagglutinin (PHA) assay, which provides a measure of the responsiveness of the adaptive immune system (Martin et al. 2006; Vinkler et al. 2010, but see Vinkler et al. 2012) and is correlated with fitness measures (Tella et al. 2002; Møller and Saino 2004; Bowers et al. 2014). Recently, we demonstrated non-random choice of extra-pair males in bluethroats, where the number of unique MHCII alleles in the pair was significantly closer to the population mean (the presumed optimum; the "golden mean") for extra-pair partners than for the social pairs in which the males had been cuckolded (Rekdal et al. 2019a). As expected, the non-random choice of extra-pair partners was reflected in the MHCII of the resulting offspring; extra-pair young (EPY) had an MHCII allele count significantly closer to the population mean than within-pair young (WPY) from the same mixed paternity broods. Furthermore, we found an elevated PHA-response in bluethroat nestlings with an MHCII allele count close to the population mean (Rekdal et al. 2021). Together, our results indicate that female bluethroats obtain offspring with an optimal MHCII allele count by engaging in extra-pair copulations.

Our finding that females with mixed-paternity broods apparently target MHC-compatible extra-pair males raises the question of whether females without EPY are able to obtain such MHC-compatible mates as social mates and hence are not in need of adjusting their initial choice by engaging in extra-pair copulations (see Fig. 1). The present study aims to answer this question, by comparing the MHCII diversity in bluethroat pairs and broods without EPY ("pure WPY-broods") with the mixed-paternity broods from Rekdal et al. (2019a), both datasets originating from the same field seasons and broods as those in Johnsen et al. (2000). Based on the hypothesis that social mates without EPY are MHC-compatible, we make the following predictions. (1) We predict that social pairs from pure WPY-broods will be closer to the population mean in their count of unique alleles within the pair, as compared to random mating. (2) We predict that the allele count in social pairs with pure WPY broods will be closer to the "golden mean" than in social pairs with mixed-paternity

**Fig. 1** Schematic illustration of the predictions in this study. Based on our previous study (Rekdal et al. 2019a), we predict that social pairs in broods without extra-pair young ("pure WPY broods") will have an MHC allele count that is centred closer around the population mean (i.e., "golden mean") than the social pairs with extra-pair young ("mixed broods"), and similar to extra-pair partners
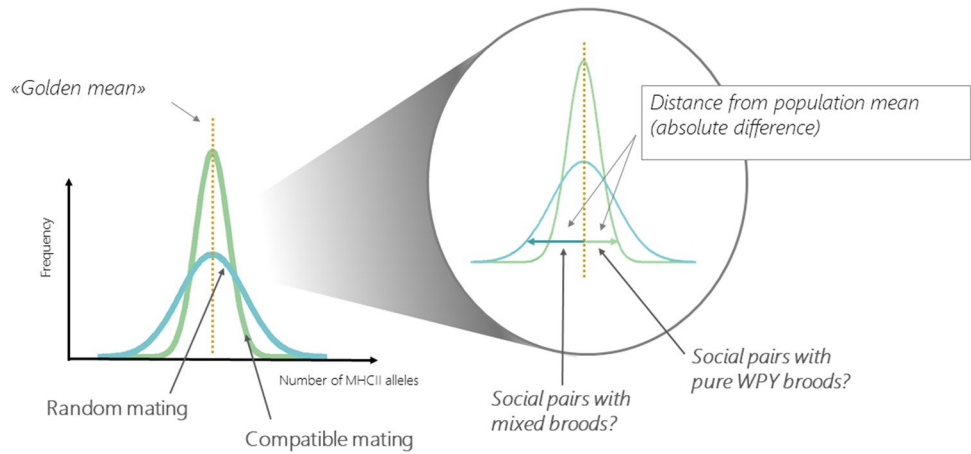


broods, and similar to extra-pair partners (see Fig. 1 and Table 1). (3) We expect to find a concurrent pattern in their offspring, with WPY from pure WPY broods having an allele count closer to the "golden mean" than WPY from mixed broods, and with similar levels as in EPY (see Fig. 1 and Table 1). However, there is a potential sexual conflict over fertilizations in bluethroats and other species in which females engage in matings with multiple males (Johnsen et al. 1998), and the outcome in terms of paternity is influenced not only by the interests of the female but also those of the social and extra-pair male(s). One aspect that may influence this sexual conflict is the age of the individuals involved (Hill et al. 1994; Birkhead et al. 1997; Graves 2004; Lifjeld et al. 2022). Specifically, the age-related patterns of sperm production and fertilization success in bluethroat males (see above) may reduce the opportunities for females paired with older males to obtain extra-pair fertilisations with more MHC-compatible males. Given that older males are better able to override female MHC preferences, we (4) predict a stronger association between MHC-compatibility and paternity success in pairs with a young male than in pairs with an older male. In other words, females should be more likely to obtain

extra-pair fertilizations when paired with a young male, but only when the male is a poor MHC match. For females, one might expect older, more experienced females to be better at selecting compatible mates than younger ones. Thus, in contrast to the situation for males, we (5) predict a stronger pattern of MHC-compatibility in pairs involving older females than those involving young females.

Finally, we also add data from the offspring in the pure WPY-broods to the dataset in which we found that offspring with an MHCII allele count close to the population mean had an increased skin-swelling response to PHA-injections (Rekdal et al. 2021), predicting (6) that the association between the PHA-response and an intermediate allele count will be reinforced as the sample size increases.

## Materials and methods

### Study population and data collection

Blood samples were collected from wild adult and nestling bluethroats in Øvre Heimdalen, Øystre Slidre, Norway (61° 25′ N, 8° 52′ E), during the breeding seasons of 1998 and

**Table 1** How we have used and defined "MHC-compatibility" and "MHC-optimality" in this study, on the levels of pairs and offspring, respectively. Our predictions and tests are made in the framework of an intermediate optimum in MHC diversity, presumably as a trade-off between pathogen recognition, and the number of circulating T cells and risk for autoimmune diseases (see text for details). The term "PSS allele" is referring to the MHC class II variants reduced to eight amino acid sites under positive selection. For pairs, the PSS allele count is based on the count of unique PSS variants when combining the genotypes of the male and female

|  | TERM | EXPLANATION FOR THE USE OF THE TERM IN THIS STUDY |
|---|---|---|
| Pairs | MHC-compatibility | The distance from the population mean (presumed optimal) count of unique PSS alleles within a pair, square root transformed. The closer the pair is to this "golden" mean in their count of unique PSS alleles, the more "MHC-compatible" the pair is |
| Offspring | MHC-optimality | The distance from population mean (presumed optimal) PSS allele count within an individual, square root transformed. The closer the individual's PSS count is to this "golden" mean, the more "MHC-optimal" the individual is |

1999 (Johnsen et al. 2000). Although the bluethroat is predominantly socially monogamous within a breeding season, about 50% of all nests contain at least one EPY (Johnsen and Lifjeld 2003). This study is based on the bluethroat nests without any EPY, to compare with the MHCII diversity of the mixed broods published in Rekdal et al. (2019a). Identity of social pairs was determined by color-banding of adults and observing territorial behavior, mate guarding, and parental care. Genetic paternity of the nestlings was determined based on microsatellite markers, previously published in Johnsen et al. (2000) and Fossøy et al. (2008). Adult birds were aged as young (second-year (SY), i.e., 1-year old) or older (after second year (ASY), i.e., 2 years or older) according to Svensson (1992).

Initially, 246 nestlings, 45 adult males and 42 adult females were included in this study, belonging to 46 pure WPY-broods (i.e., broods with no EPY. See online resource S1). One male was sampled and identified as the within-pair male (WPM) in both 1998 and 1999. Females of four broods were not successfully sampled, but still paternity could be verified. All social pairs (i.e., the combination of female and male identities) were unique.

## Sequencing and allele calling of MHCIIβe2

The second exon of the MHCII β-chain (MHCIIβe2) was amplified in duplicates from the DNA extracted from the blood samples and sequenced on an Illumina MiSeq® machine. Allele calling was done according to a modification of the pipeline established in Rekdal et al. (2018), which was based on the workflow described in Sommer et al. (2013). The pipeline utilizes read depths, replicate runs, and family information to detect alleles and artifacts. For the statistical tests, genotypes based on eight positively selected amino acid sites (PSS) were used, in order to restrict the analyses to the putative antigen-binding sites only. We also clustered the PSS alleles into supertypes, based on their physiochemical properties (Sandberg et al. 1998). For details on the sequencing and genotyping, see online resource S18.

## PHA assay

The nestlings were initially injected subcutaneously with 0.10 mg phytohaemagglutinin (PHA-P, dissolved in 40 µL saline) in the metacarpal region of the right wing at day 5 post-hatching (Johnsen et al. 2000). At day 7, they received a second injection in the ulnar region of the same wing. Injections of 40-µL saline in the left wing at both days served as control. We used a spessimeter (Teclock SM-112) to measure wing thicknesses before and $24 \pm 1$ h after the two

injections. Skin swelling was estimated as the change in the thickness of the right wing minus the change in the thickness of the left wing for the injections at day 5 and day 7 separately. All measurements were repeated and averaged, with high repeatability (0.96–0.98; Johnsen et al. 2000). Skin-swelling measurements were done blindly with respect to the paternity of the nestlings.

## Data analyses

Our analyses aimed to test the hypothesis that females without EPY are socially paired with an MHC compatible mate. The tests were run in the framework of an intermediate optimum in MHC allele count, where the optimal number of PSS alleles is assumed to be close to the population mean. This is in line with the previously published results in bluethroats (Rekdal et al. 2019a, 2021), as well as in three-spined sticklebacks (Aeschlimann et al. 2003). "MHC-optimality"/"MHC-compatibility" was therefore established as the difference from the population mean (the optimum, or "golden mean") in the individuals' or pairs' PSS allele count (square root transformed to achieve normality, as in Rekdal et al. (2019a); see Table 1).

We tested if the MHC-compatibility in social pairs with pure WPY broods (hereafter termed WPM-F$_{pure}$, i.e., within-pair male-female$_{pure}$) deviated from random pairs by comparing the observed mean count of unique PSS alleles in the pairs against 10,000 means across simulated, random pairs. Further, we expected WPM-F$_{pure}$ to be more MHC-compatible than social pairs from mixed broods (WPM-F$_{mixed}$), but similar to extra-pair partners (EPM-F). We tested differences in MHC-compatibility among these groups using Welch's $t$ tests, using also the data from Rekdal et al. (2019a, b). An association between MHC-compatibility in the social pair and the presence of at least one EPY in the brood was tested using binomial generalized linear models.

Similarly, we expected WPY from pure WPY-broods (WPY$_{pure}$) to be more MHC-optimal than WPY from mixed broods (WPY$_{mixed}$), but similar to EPY in such broods. This was tested using linear mixed models with the parents' identity as random factor with random intercepts. As for the adults, an association between MHC-optimality in the WPY and the presence of any EPY in the brood was tested using binomial generalized linear models. All analyses were conducted on five datasets: one including all individuals, and then separately for each male and female age category (young/older). Accordingly, we also tested for assortative mating based on age (young and older males and females) for social pairs, using Fisher's exact test.

With the exception of the simulation, we also ran corresponding tests on the level of supertypes. For details on the data analyses, see online resource S20.

Many of the nestlings from the pure WPY-broods ($n = 158$) had their skin-swelling measured following two injections of PHA (at day 5 and day 7), as in the dataset published in Rekdal et al. (2021). We thus ran similar linear mixed models as in Rekdal et al. (2021) on the present dataset of pure WPY-broods, testing the correlations between the skin-swelling response (dependent variable) and the distance from the optimal PSS allele count (square root transformed; predictor variable). As for the analyses in Rekdal et al. (2021), mean ambient temperature during the nestling period, the body mass at the day of the injection, and the body mass and tarsus length of the female of the brood were also added as fixed effects. We used brood identity as random effect with random intercepts. The models were run for the skin-swelling responses following the two PHA injections separately. Additionally, similar linear mixed models were run with the predictor variable divided in a centred within-brood variable and an averaged among-brood variable (van de Pol and Wright 2009). We also ran the above tests on the combined dataset of all offspring (both mixed paternity broods and pure WPY-broods), including also paternity as fixed effect.

All statistical analyses were run in R v4.0.2 (R Core Team 2020), using the packages tidyverse (Wickham et al. 2019), lmerTest (Kuznetsova et al. 2017), reshape (Wickham 2007), scales (Wickham and Seidel 2022), ggpubr (Kassambara 2020) car (Fox and Weisberg 2018), and effsize (Torchiano 2020). The R-script is available in online resource S17. The main level for analyses presented in this study is the PSS alleles, but corresponding results on the supertype level are given in online resource S16. We controlled for prediction-wide multiple testing using the false discovery rate approach described in Benjamini and Hochberg (1995; see online resource S15).

## Results

The final dataset included 302 individual bluethroats (38 adult females, 43 adult males, and 221 nestlings) from 43 pure WPY-broods, of which 38 were complete with both the adult female, adult male, and nestlings successfully genotyped. Across all individuals, 376 unique MHCII PSS alleles were scored in at least one individual. The number of MHCII PSS alleles per individual ranged from 13 to 46, with a population mean of 25.2 over all adults sampled in 1998 (SD: 5.60, $n = 34$), and 24.6 over all adults sampled in 1999 (SD: 5.84, $n = 47$). For the observed pairs (i.e., the social male and the female of a pure WPY-brood (WPM-F$_{pure}$)), the mean count of unique PSS alleles per pair was 41.8 in 1998 (SD: 7.57, $n = 16$ pairs) and 40.5 in 1999 (SD: 6.31, $n = 22$ pairs).

### Tests of deviation from random pairing

We compared the mean number of unique PSS alleles within social pairs with pure WPY-broods (WPM-F$_{pure}$) to the distribution based on 10,000 rounds of simulations, where one male was randomly drawn (without replacement) as the WPM for each of the sampled females ($n = 38$) in every round of simulation. The mean value for all observed pairs was not significantly closer to the optimum than expected from random pairing, as 2159 of the 10,000 simulated means were closer to the optimum than the mean across the observed WPM-F$_{pure}$ (exact test: $P = 0.43$; Fig. 2).

When including only the females that were socially paired with a young male ($n = 12$), the observed mean count of unique PSS alleles per pair was significantly closer to the optimum than random pairs across all females ($P = 0.0012$; significant also after controlling for multiple testing, see online resource S15). The observed mean count of unique PSS alleles per pair for pairs with older WPM only ($n = 26$) was not significantly closer to the optimum than random pairs ($P = 0.68$; Fig. 2). The observed mean count of unique PSS alleles per pair was also not significantly different from random when including only young or older females separately (young: $P = 0.35$, $n = 14$, older: $P = 0.15$, $n = 23$).

### Comparisons with mixed broods: pairs

Including all pairs, social pairs with pure WPY-broods (WPM-F$_{pure}$) were not more MHC-compatible than social pairs with EPY (WPM-F$_{mixed}$; $t_{71.9} = -1.56$, $P = 0.12$). There was also no difference in MHC-compatibility between WPM-F$_{pure}$ and extra-pair partners (EPM-F; $t_{67.2} = 0.87$, $P = 0.39$; Fig. 3).

The test including the pairs with young males only showed a tendency for WPM-F$_{pure}$ to be more MHC-compatible than WPM-F$_{mixed}$ ($t_{17.6} = -1.95$, $P = 0.068$; effect size $= 0.85$ (95% CI $-0.11$–$1.82$); Fig. 3), while there was no difference between WPM-F$_{pure}$ and EPM-F ($t_{4.63} = -0.40$, $P = 0.71$; Fig. 3). The tests including only older males showed no differences among the groups (WPM-F$_{pure}$ vs WPM-F$_{mixed}$: $t_{50.9} = -0.54$, $P = 0.59$; WPM-F$_{pure}$ vs EPM-F: $t_{45.4} = 1.43$, $P = 0.16$). Similarly, the tests including pairs with young females showed that WPM-F$_{pure}$ were more MHC-compatible than WPM-F$_{mixed}$ ($t_{23.3} = -2.39$, $P = 0.025$; effect size $= 0.93$ (95% CI 0.094–1.76); online resource S21), but this result was not significant after correction for multiple testing (online resource S15). There was no difference between WPM-F$_{pure}$ and EPM-F ($t_{24.7} = 0.085$, $P = 0.93$; online resource S21). Further, there were no differences among these groups when including only pairs with older females (WPM-F$_{pure}$ vs WPM-F$_{mixed}$: $t_{43.4} = -0.72$,

**Fig. 2** Distribution of 10,000 means of the distance from optimal count of unique PSS alleles (i.e., absolute difference from the "golden mean" located at $x = 0$) within a pair across all bluethroat females without any extra-pair young (square root transformed; green bars), where the mean values are calculated by randomly drawing one available male for each of the 38 females from the pure WPY-broods. The mean value across the observed social pairs (WPM-$F_{pure}$) is added as the green, dashed line, while the mean values across the observed pairs with young (SY) males/old (ASY) males/young (SY) females/old (ASY) females are added as gray, orange, light pink, and dark pink lines, respectively. Density plot (shaded green) from the simulation including all possible pairs is added for comparison
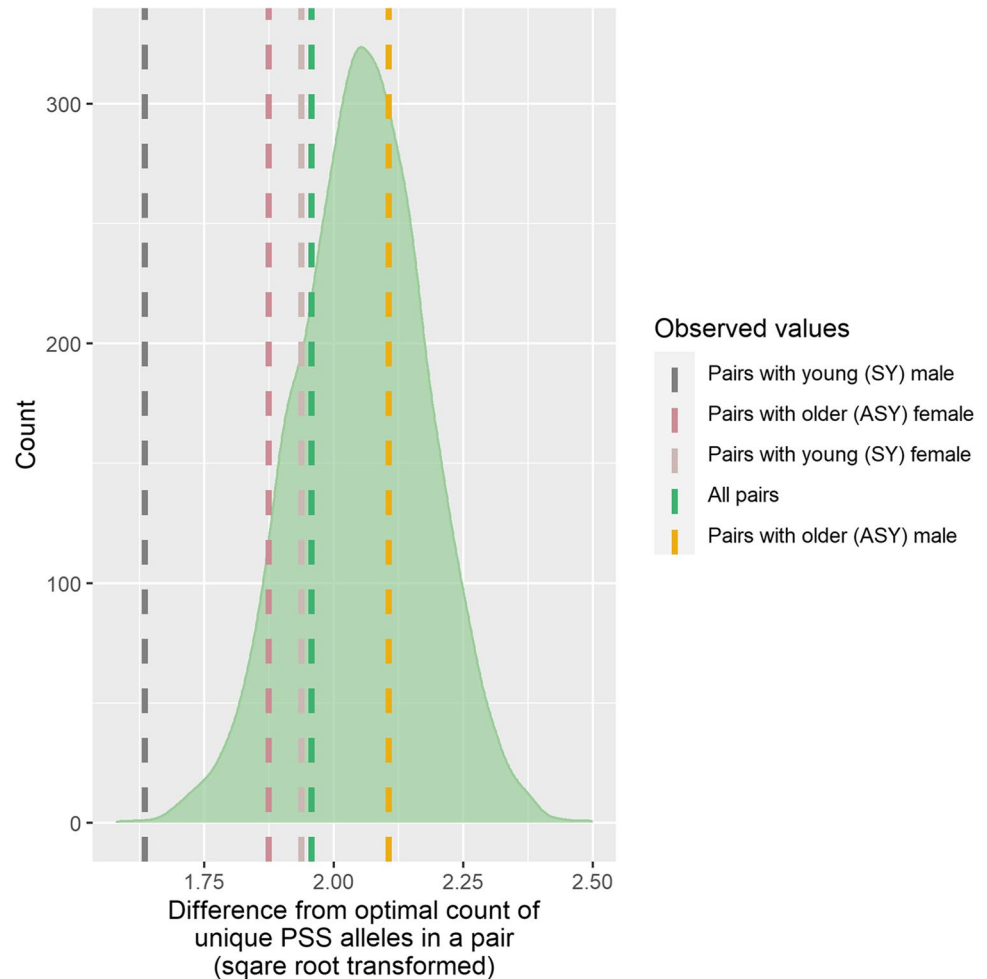


The binomial generalized linear model showed the same pattern using the presence/absence of EPY as response variable; there was no association between MHC-compatibility in the social pairs and the presence of at least one EPY in the brood when including all pairs (estimate = − 0.39, SE = 0.26, $P = 0.12$), nor when including pairs with older males (estimate = − 0.17, SE = 0.31, $P = 0.58$) or older females (estimate = -0.23, SE = 0.31, $P = 0.46$) only. When including only pairs with young males, MHC-compatible pairs tended to not have any EPY in the brood (estimate = -0.89, SE = 0.50, $P = 0.078$). This association was significant for pairs including young females only (estimate = − 1.21, SE = 0.58, $P = 0.037$; see online resource S22), but turned non-significant when controlling for multiple testing (online resource S15).

No tests were significant on the supertype level (online resource S16), but there was a tendency for WPM-$F_{pure}$ to be more MHC-compatible than WPM-$F_{mixed}$ ($t_{53.6} = − 1.84$, $P = 0.072$) and MHC-compatible pairs to not have any EPY in the brood (estimate = − 1.09, SE = 0.60, $P = 0.071$).

## Comparisons with mixed broods: offspring

The linear mixed model showed no significant difference in MHC-optimality between WPY$_{pure}$ and WPY$_{mixed}$ (linear mixed model: estimate = − 0.18, SE = 0.12, $P = 0.14$; Fig. 4), nor between WPY$_{pure}$ and EPY (estimate = 0.13, SE = 0.12, $P = 0.27$; Fig. 4). A clearer pattern was found when including only broods with young sires; WPY$_{pure}$ were more MHC-optimal than WPY$_{mixed}$ (estimate = − 0.52, SE = 0.21, $P = 0.019$; borderline significant after controlling for multiple testing, see online resource S15), but not more MHC-optimal than EPY (estimate = 0.45, SE = 0.31, $P = 0.15$). No differences were found among the groups when including only broods with older sires (WPY$_{pure}$ versus WPY$_{mixed}$: estimate = − 0.065, SE = 0.14, $P = 0.65$; WPY$_{pure}$ versus EPY: estimate = 0.13, SE = 0.14, $P = 0.34$). Likewise, WPY$_{pure}$ were more MHC-optimal than WPY$_{mixed}$ when including only the broods with a young female (estimate = − 0.45, SE = 0.19, $P = 0.028$, also borderline significant after controlling for multiple testing, see online resource S15 and online resource S23), but not more MHC-optimal than EPY

**Fig. 3** Boxplots of the distance from the golden mean in PSS allele count for social pairs (WPM-F) from broods without any extra-pair young (i.e., pure WPY-broods: WPM-F$_{pure}$, sequenced in this study), and for social pairs and extra-pair partners from broods with at least one extra-pair young (i.e., mixed broods: WPM-F$_{mixed}$ and EPM-F, from Rekdal et al. (2019a)). The data are divided based on the age of the pair male (young: SY = second year, older: ASY = after second year). The individual observations are added as jittered points (black and gray circles). The allele count is scaled to the data for the mixed broods, published in Rekdal et al. (2019a). The figure shows non-square root transformed data, to make it comparable to Fig. 3 in Rekdal et al. (2019a). Boxes indicate the inter quartile range (IQR), with the central line depicting the median and the whiskers extending to the largest/smallest values within 1.5*IQR
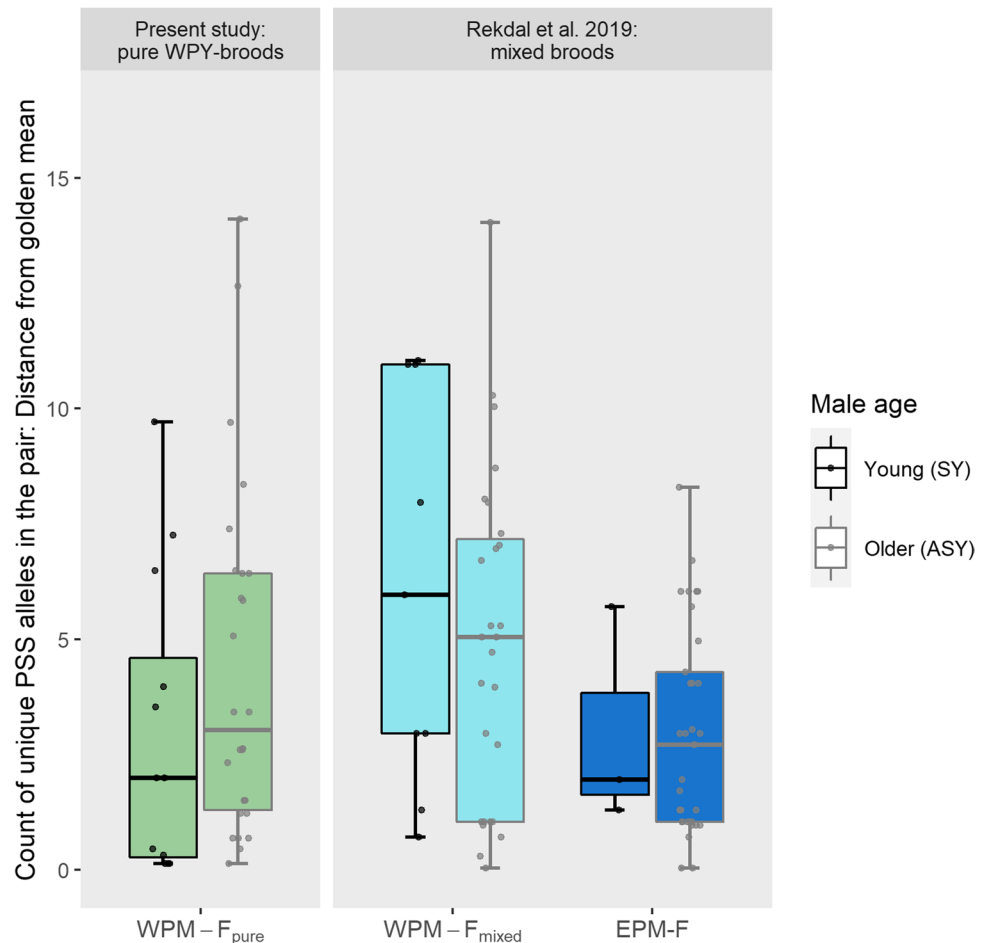


(estimate = 0.044, SE = 0.19, $P$ = 0.82). There were no differences between the groups with an older dam (WPY$_{pure}$ versus WPY$_{mixed}$: estimate = − 0.071, SE = 0.16, $P$ = 0.66; WPY$_{pure}$ versus EPY: estimate = 0.14, SE = 0.16, $P$ = 0.40).

There was no significant association between the MHC-optimality in the WPY in a brood, and the presence/absence of at least one EPY (estimate = − 0.62, SE = 0.44, $P$ = 0.16). However, the association of being MHC-optimal when there was no EPY in the brood was significant when including only WPY with young sires/dams (young sires: estimate = − 2.75, SE = 1.29, $P$ = 0.033, young dams: estimate = − 1.89, SE = 0.92, $P$ = 0.039, both significant after controlling for multiple testing, see online resource S15). This was not significant when including WPY with older parents (older sires: estimate = − 0.16, SE = 0.49, $P$ = 0.75, older dams: estimate = − 0.31, SE = 0.60, $P$ = 0.60). See online resource S24.

For the supertypes, there was a tendency for WPY$_{pure}$ to be more MHC-optimal than WPY$_{mixed}$ (estimate = − 0.15, SE = 0.078, $P$ = 0.064), with the tendency only being present when analyzing offspring with young parents (young sires: estimate = − 0.22, SE = 0.12, $P$ = 0.082; young dams: estimate = − 0,26, SE = 0.14, $P$ = 0.068; older sires:

estimate = − 0.13, SE = 0.098, $P$ = 0.19; older dams: estimate = − 0.037, SE = 0.10, $P$ = 0.72). See online resource S16.

## Assortative mating among social pairs

There was a significant age-based assortative mating in social pairs (Fisher's exact test, $P$ = 0.0075), with a pattern of young females mainly pairing with young males, and older females mainly pairing with older males. For the age distribution in social pairs (both WPY-broods and mixed broods (Rekdal et al. 2019a)), see Table 2.

## PHA and MHCII in pure WPY broods

The linear models including only the pure WPY-broods did not support the main finding of a correlation between the skin-swelling response to injections of PHA and MHC-optimality from Rekdal et al. (2021; all $P$ > 0.17). The skin-swelling following the initial PHA injection was however positively associated with the nestlings' body mass (estimate = 0.040, $P$ ≤ 0.001; see online resource S13) and negatively associated with the ambient temperature (estimate = − 0.080, $P$ ≤ 0.012; see online resource S13).

**Fig. 4** Boxplots of the distance from the optimal PSS allele count for within-pair young (WPY) from broods without any extra-pair young (i.e., pure WPY-broods: $WPY_{pure}$, sequenced in this study), and for WPY and extra-pair young from broods with at least one extra-pair young (i.e., mixed broods: $WPY_{mixed}$ and EPY, from Rekdal et al. (2019a)). The data is divided based on the age of the pair male (young: SY = second year, older: ASY = after second year). The allele count is scaled to the data for the mixed broods, published in Rekdal et al. (2019a). The individual observations are added as jittered points (black and gray circles). Boxes indicate the inter quartile range (IQR), with the central line depicting the median and the whiskers extending to the largest/smallest values within 1.5*IQR
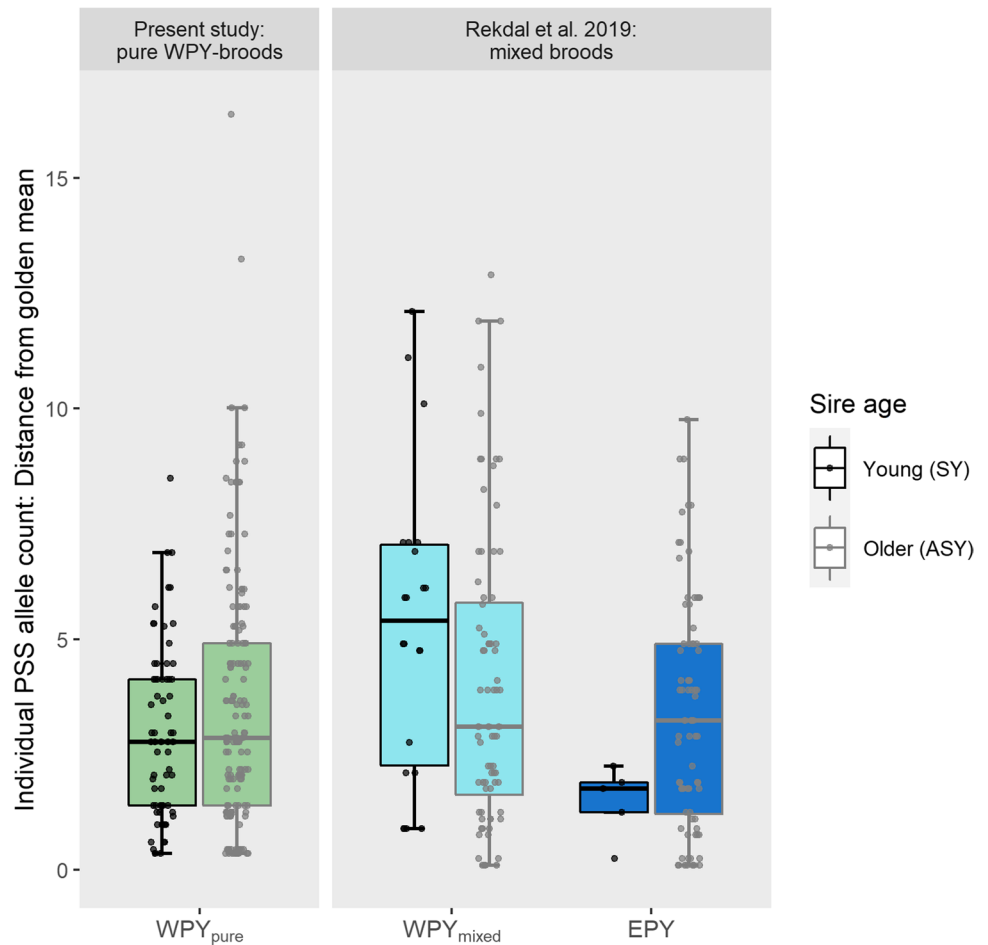


**Table 2** The number of broods included within the different combination of male and female age for the social pairs, for both pure WPY-broods (WPM-$F_{pure}$) and mixed broods (WPM-$F_{mixed}$). The age is divided in two groups: young (second year; hatched the previous year) and older (above second year). One female in a WPM-$F_{pure}$ pair was not age determined (paired to an older male)

| Female age | Male age | $N$ (WPM-$F_{pure}$) | $N$ (WPM-$F_{mixed}$) |
|---|---|---|---|
| Young | Young | 7 | 6 |
| Young | Older | 7 | 7 |
| Older | Young | 5 | 3 |
| Older | Older | 18 | 20 |

The correlation with the nestlings' body mass was also found after the second PHA injection (estimate $\geq 0.050$, $P \leq 0.010$), but no other significant correlations were found.

Combining the present dataset with the dataset of the mixed broods (Rekdal et al. 2019a, 2021) reinforced the finding that nestlings with a PSS allele count close to the population mean had an increased skin swelling response to the first PHA-injection. This was mainly due to a within-brood effect (estimate = $-0.051$, SE = 0.020,

$P = 0.0097$; see Table 3). After the second PHA injection, there was a significant among-brood effect (estimate = $-0.17$, SE = 0.071, $P = 0.018$; see Table 3). There was also a significant effect of paternity and brood type after the second injection, where EPY had the largest swelling response (estimate $\leq 0.15$, $P < 0.024$). Running pairwise models (see online resource S13) revealed that EPY had a significant larger skin-swelling response at day 7 than both WPY from pure WPY-broods (estimate = $-0.25$, $P \leq 0.0011$) and WPY from mixed broods (estimate $\leq -0.15$, $P \leq 0.039$). There was no difference in this response between WPY from pure WPY-broods and WPY from mixed broods (estimate $\leq -0.054$, $P \geq 0.48$).

## Discussion

We found that social pairs with pure WPY-broods with a young male were significantly more MHC-compatible than expected from random pairing. There was also a tendency for social pairs without EPY to be more MHC-compatible than social pairs with EPY in their broods, but only in the subset of pairs involving a young male and/or female. A

**Table 3** Full-linear models testing correlations between the skin-swelling following injections of phytohaemagglutinin (PHA) and the distance to the population mean number of PSS alleles (MHCII; square root transformed) in bluethroat nestlings, originating from broods with and without extra-pair young (EPY). The nestlings are grouped in three groups: WPY from pure WPY-broods (WPY(WPY_brood)), and WPY and EPY from mixed broods (WPY (mixedBroods) and EPY (mixedBroods), respectively). Nestling body mass (body mass$_{ind}$), mean ambient temperature during nestling period (temperature), and the body mass and tarsus length of the adult female of the brood were additionally entered as fixed effects, while brood identity was used as random factor with random intercepts. The models were run for the skin-swelling following the initial and the second PHA-injections separately, and both with and without a within-brood centring of the MHCII variable. P values < 0.05 are shown in bold

| Fixed effects | Skin swelling following initial PHA injection (day 5) | | | | | | | | | | Skin swelling following second PHA injection (day 7) | | | | | | | | | |
| | No within-brood centring of MHCII variable | | | | | Within-brood centring of MHCII variable | | | | | No within-brood centring of MHCII variable | | | | | Within-brood centring of MHCII variable | | | | |
| | Estimate | Std error | df | t | P | Estimate | Std error | df | t | P | Estimate | Std error | df | t | P | Estimate | Std error | df | t | P |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Intercept | 0.33 | 0.22 | 60.1 | 1.49 | 0.14 | 0.35 | 0.22 | 57.3 | 1.56 | 0.12 | −0.049 | 0.46 | 74.9 | −0.11 | 0.92 | 0.10 | 0.44 | 74.5 | 0.23 | 0.82 |
| MHCII | −0.056 | 0.018 | 253.0 | −3.19 | **0.0016** | | | | | | 0.0095 | 0.032 | 265.4 | 0.30 | 0.77 | | | | | |
| MHCII$_{centred}$ | | | | | | −0.051 | 0.020 | 207.5 | −2.61 | **0.0097** | | | | | | 0.048 | 0.035 | 217.8 | 1.38 | 0.17 |
| MHCII$_{broodMean}$ | | | | | | −0.076 | 0.040 | 51.2 | −1.92 | 0.060 | | | | | | −0.17 | 0.071 | 53.7 | −2.44 | **0.018** |
| Paternity$_{EPY\text{-}WPY(mixedBroods)}$ | 0.031 | 0.036 | 238.9 | 0.87 | 0.39 | 0.030 | 0.036 | 238.2 | 0.85 | 0.40 | −0.15 | 0.065 | 246.2 | −2.28 | **0.024** | −0.15 | 0.064 | 249.0 | −2.35 | **0.020** |
| Paternity$_{EPY\text{-}WPY(WPY\_broods)}$ | 0.0011 | 0.042 | 82.1 | 0.03 | 0.98 | −0.00034 | 0.043 | 79.8 | −0.01 | 0.99 | −0.22 | 0.081 | 78.7 | −2.68 | **0.0090** | −0.22 | 0.078 | 82.3 | −2.86 | **0.0054** |
| Body mass$_{ind}$ | 0.046 | 0.0093 | 185.5 | 4.96 | **0.0000016** | 0.047 | 0.0093 | 186.6 | 4.99 | **0.0000014** | 0.041 | 0.017 | 248.6 | 2.43 | **0.016** | 0.045 | 0.017 | 242.3 | 2.71 | **0.0073** |
| Temperature | −0.053 | 0.025 | 51.1 | −2.15 | **0.036** | −0.053 | 0.025 | 50.1 | −2.17 | **0.035** | 0.094 | 0.048 | 51.0 | 1.97 | 0.054 | 0.092 | 0.045 | 51.9 | 2.04 | **0.046** |
| Body mass$_{female}$ | −0.0031 | 0.019 | 55.0 | −0.17 | 0.87 | −0.0028 | 0.019 | 54.0 | −0.15 | 0.88 | −0.024 | 0.037 | 55.3 | −0.64 | 0.53 | −0.018 | 0.035 | 56.8 | −0.52 | 0.61 |
| Tarsus length$_{female}$ | 0.0062 | 0.012 | 55.2 | 0.503 | 0.62 | 0.0064 | 0.012 | 53.8 | 0.52 | 0.60 | 0.013 | 0.024 | 55.5 | 0.52 | 0.61 | 0.014 | 0.023 | 56.8 | 0.59 | 0.56 |
| Random effect | Variance | Standard deviation | | | | Variance | Standard deviation | | | | Variance | Standard deviation | | | | Variance | Standard deviation | | | |
| Brood | 0.011 | 0.10 | | | | 0.011 | 0.10 | | | | 0.046 | 0.21 | | | | 0.039 | 0.20 | | | |
| Residual | 0.031 | 0.18 | | | | 0.031 | 0.18 | | | | 0.10 | 0.32 | | | | 0.102 | 0.32 | | | |

corresponding pattern was evident in their offspring: WPY from pure WPY-broods were more MHC-optimal than WPY from mixed broods, in broods with at least one young parent. Taken together with our previous demonstration that extra-pair mates and EPY are more MHC-compatible than social mates and WPY in broods with mixed paternity (Rekdal et al. 2019a), our current results support the hypothesis that mate choice is associated with MHCII compatibility in the bluethroat. The pattern of an increased skin-swelling following PHA-injections at an intermediate MHC allele count (Rekdal et al. 2021) was reinforced when adding the dataset of pure WPY broods, although it was not statistically significant for the latter analysed separately.

Why would only females with a young male be able to realise their preference for a mate that gives rise to offspring with intermediate levels of MHCII variability? One possibility is that older males are more proficient at obtaining full paternity in their broods, even when they are not optimal with respect to MHCII, e.g., due to their higher sperm number and/or experience with copulations (Johnsen et al. 2001; Laskemoen et al. 2008). Hence, even though females may have preferences for males with a complementary constitution of MHCII, the interests of the pair males may sometimes counteract the females' preferences. This may be more likely when the male is old, as older males have considerably larger testes than young males (38%, Laskemoen et al. 2008), which ultimately is an advantage for obtaining full paternity. In other words, it is possible that social pairing with a young male producing a pure WPY-brood reflects a compatibility situation, where the social male is the best match and there is no sexual conflict over paternity. In the group of pairs in which the male is older there will be some that are not compatible at the MHCII, but where the male still obtains full paternity. This could explain the large variation in the distance to the optimum for pairs involving older males (see Fig. 3). Since we do not know the patterns of copulations, we cannot know whether females with pure WPY-broods have copulated only with their social male or with several males without any fertilization success. Based on a previous study, in which pair males were prevented from inseminating their mates, it seems likely that most female bluethroats engage in multiple mating under given circumstances (Fossøy et al. 2006), implying that many of the females with pure WPY-broods probably have copulated with multiple males.

Our predictions were only supported within the relatively small subgroup of pairs involving young males. It should be noted, however, that the pattern was evident in two independent sets of analyses. First, we found non-random pairing with respect to the combined MHCII diversity in pairs involving young males, with such pairs being more MHC-compatible than expected from random pairing. Second, in the comparisons of social mate choice in pure WPY-broods and broods with mixed paternity, there was a tendency in the predicted direction for pairs involving young males and a concomitant significant difference between offspring with a young sire in pure WPY-broods and WPY from mixed broods. In sum, our results suggest that male age interacts with MHC-compatibility to shape the outcome of social mate choice in terms of paternity in the bluethroat. There were also significant effects of female age in some of our analyses. As there is a strong age-based assortative mating in the bluethroat (AJ and JTL, unpublished data, this study), we believe that the patterns observed when analyzing the subset of young females are driven by the age-specific patterns for the males. This is also supported by the tests of deviation from random mating, where the effect was found in pairs with young males only, and not in pairs with young females.

The mechanism by which females are able to distinguish MHC-compatible from MHC-incompatible males remains unknown. A pre-copulatory mate choice based on odor profiles has been demonstrated in other birds (Leclaire et al. 2014, 2017; Slade et al. 2016; Grieves et al. 2019). However, given our previous result suggesting that most females engage in EPCs in this species (Fossøy et al. 2006), a post-copulatory (cryptic) female choice at the level of gametes or reproductive fluids is also likely. Post-copulatory choice by haploid gametes offers the possibility of a more precise choice of MHC-haplotypes than pre-copulatory mate choice in diploid individuals allows for (Lenz et al. 2018; Milinski 2022). Lenz et al. (2018) did indeed find that stickleback gametes combined in a non-random manner with respect to MHC, yielding offspring that were more MHC-optimal than random fusion of gametes from their parents would predict. Here, we found stronger statistical support at the offspring level than at the level of their parents, which could indicate that some form of haplotype matching at the gamete level is at play also in the bluethroat. However, we are currently not able to analyse linkage of alleles into haplotypes in the extremely diverse bluethroat MHCII. Revealing the genomic organization of the MHCII loci in the bluethroat, and the co-segregation of alleles in linkage groups, offer an interesting avenue for future research.

Whether an intermediate MHCII diversity relates positively to fitness remains to be shown conclusively. When combining our present dataset with the dataset from Rekdal et al. (2021), we found that offspring that had closer to the mean number of MHCII PSS alleles mounted a stronger PHA response than offspring at either end of the distribution, with a significant within-brood effect after the first injection and a significant among-brood effect after the second injection. The results from Rekdal et al. (2021) were thus strengthened when adding the present dataset, even though there were no significant effects in this dataset alone. The relationship between the PHA response and fitness is

debated (e.g., Vinkler et al. 2012; Bowers et al. 2014). Based on the available evidence for the bluethroat, with extra-pair offspring showing a higher PHA response than their half-siblings (Johnsen et al. 2000; Fossøy et al. 2008) and an additional positive association with intermediate MHCII diversity (Rekdal et al. 2021, this study), it seems likely that a high PHA-response is positive for the individual and thus related to fitness. Nevertheless, future studies should focus on other fitness traits to test the hypothesis that an intermediate diversity of MHCII is selected for in this and other species.

In conclusion, we found evidence for the expectation that female bluethroats without extra-pair young in their broods have an MHC-compatible social mate, but only within the subgroup of females that are mated to young males. This could be explained by the higher sperm competitiveness of older males, essentially overriding female preferences. Thus, our results indicate that mate choice for compatible MHC-genes operates in a context of sexual conflict, where age-specific characteristics of the individual players affect the outcome.

## Declarations

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