Female reed warblers in social pairs with low MHC dissimilarity achieve higher MHC compatibility through random extra-pair matings

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Abstract

Major Histocompatibility Complex (MHC) polymorphism is maintained by balancing selection through host-pathogen interactions and mate choice. The likelihood of mate choice based on MHC-dissimilarity in songbirds has been questioned because of their rather poorly developed olfactory sense, a trait considered crucial in pre-copulatory mate choice to determine both own MHC and MHC of putative partners. However, our results suggest a mechanism where extra-pair matings can maintain high MHC polymorphism also without the need for olfactory cues. In our study population of reed warblers, *Acrocephalus scirpaceus,* females with extra-pair young in their broods have a higher MHC similarity with their social mate than females without extra-pair young in their broods. We suggest that when MHC similarity is high between social mates, females can improve MHC diversity in their offspring through extra-pair mating. A post-copulatory mate choice mechanism that relies on multiple copulations does not demand that females identify and actively choose MHC-dissimilar mating partners. In line with this reasoning the MHC-dissimilarity of successfully siring extra-pair males were neither different from that of social pairs without extra-pair young nor from putative extra-pair males that were breeding close to the female's social nest.

Introduction

The potential role of the Major Histocompatibility Complex (MHC) in mate choice has intrigued evolutionary biologists and behavioural ecologists for decades [1–4]. MHC molecules are central for antigen presentation and T-cell mediated adaptive immunity [5]. MHC genes are highly polymorphic and maintained by selection from pathogens and / or by mate choice [4,6,7]. An optimally high MHC diversity (number of different MHC alleles per individual), or MHC divergence (functional distance between MHC alleles within an individual), is advantageous since a wider range of pathogens can be detected and eliminated, and studies have shown that MHC diversity / divergence correlate with, disease resistance, increased survival and fitness in wild populations [8–11]. In order to maximize offspring MHC diversity / divergence mate choice for MHC-dissimilar partners is predicted [9–11].

A large number of studies have investigated if social mate choice is MHC-based (MHC-dissimilarity, MHC-diversity and specific MHC genotypes), using a plethora of study systems, techniques and approaches [1,3,12–16]. In general, MHC-based social mate choice appears to be fairly infrequent among vertebrates [2,17,18]. However, social mate choice is strongly influenced by direct benefits, such as territory quality, social rank, and ability to provision for offspring, which may override or obscure the signal of MHC-based mate choice, an indirect benefit [19–22]. Extra-pair mate choice in contrast, *i.e.,* mating outside the social pair-bond, is largely free of such constraints and therefore provides an excellent model for investigating MHC-based mate choice [2,23]. Indeed, extra-pair mate choice for MHC-dissimilar partners could partly compensate for high MHC-similarity within the social pair.

Songbirds are well suited to studies of extra-pair mate choice as although most species are

socially monogamous, extra-pair mating is common [24–26]. However, many songbird species have highly duplicated MHC genes, which has previously made obtaining accurate MHC genotypes challenging [27, 28]. Since the advent of next-generation sequencing, the use of high-throughput amplicon sequencing (HTS) has enabled the detailed characterization of MHC genes even in species with extreme MHC gene copy numbers [29, 30]. A handful of studies have used HTS approaches to study the role of MHC in mate choice in songbirds with mixed results [14,27,28]. Therefore, many open questions surrounding the role of MHC genes in mate choice in songbirds remain, such as whether extra-pair mating compensate for suboptimal social mate choice in relation to MHC-dissimilarity.

Here we test whether MHC dissimilarity plays a role in social and extra-pair mate choice in a wild study population of reed warblers, *Acrocephalus scirpaceus,* in Stawy Milickie nature reserve in South-West Poland. Reed warblers are socially monogamous but have a high frequency of successful extra-pair mating (up to 33% of breeding females have at least one extra-pair chick in their brood) [25]. We combined field observations of breeding pairs over six years (128 females and 127 males) with data on their MHC genotypes to explore the relationship between MHC dissimilarity within social pairs, the occurrence of extra-pair paternity and the choice of extra-pair male. This extensive dataset allowed us to go beyond previous studies by demonstrating how the choice of extra-pair male relates to the locally available males in terms of MHC-dissimilarity. In doing so we present compelling evidence for how extra pair mating can compensate for poor within-pair MHC dissimilarity even in the absence of active extra-pair mate choice.

Methods

100 *Study population*

101 Reed warblers are socially monogamous migratory passerine birds [29]. Most social pairs
102 remain together throughout a breeding season (May – August) laying up to four clutches, but
103 disband between seasons [25,30]. Both males and females are heavily engaged in both
104 incubation and feeding young [30]. Each female generally has only a few unmated males to
105 choose from when seeking a social mate. However, there are often many male neighbours in
106 the vicinity of any given female's nest, providing opportunities for extra-pair mating. During
107 May to August of 2006 to 2011, we studied a population of reed warblers breeding in the
108 nature reserve "Stawy Milickie", SW Poland (51°32 N, 17°20 E). We monitored the study
109 plot daily from the time the first individuals arrived and mist-netted and individually marked
110 birds throughout the breeding season. Each year we monitored between 29 and 51 breeding
111 pairs, as well as putative extra-pair males around each nest.

112

113 *DNA extraction*

114 Each adult was sexed and a blood sample (10–40µl) was taken (288 individuals). Nestlings
115 were blood sampled when 6-8 days old (501 chicks). Blood samples were preserved in 96%
116 ethanol and kept in a freezer until DNA extraction using DNeasy Blood and Tissue Kit
117 (QIAGEN, Hilden, Germany) or GeneMATRIX Tissue DNA Purication Kit (EURx, Gdańsk,
118 Poland). The DNA extracts were kept frozen at -20°C until used (QIAGEN, Hilden,
119 Germany).

120

121 *MHC genotyping*

122 We prepared forward and reverse 6bp-tagged amplicon libraries for high-throughput 454
123 sequencing of exon 3, the most variable fragment of the gene encoding the antigen binding
124 site of the MHC molecule, from 293 adult individuals (of which 24 individuals were

duplicated to aid with filtering the data) using the forward primer HN36 and reverse primer HN46. These primers were previously designed to amplify MHC-I alleles in a closely related species, the great reed warbler *Acrocephalus arundinaceus* [31] and we ran PCRs as described in [9]. The 454-sequencing data (312 amplicons, 46471 reads) was filtered to remove low-quality amplicons and sequences, *i.e.*, sequences representing artefactual MHC alleles and potentially non-functional alleles as described in [9]. The minimum total sequence abundance/individual (filter 1) was set to 75 reads and the minimum abundance of one sequence in all individuals (filter 2) was set to 4 reads. The 24 technical duplicates were used to determine the threshold for minimum relative abundance of a sequence/individual (filter 3). This filter was set to 6% (based on best between-duplicate identity: 95% total similarity). Any alleles not found in at least two independent PCR reactions were removed. Sequences were inspected manually and any non-functional sequences (four) or chimeras (two) were removed. After filtering, the final data set contained 281 sequences found in 255 individuals (128 females and 127 males) among which there were 141 breeding pairs (15 to 31 pairs per year). The average number of reads per amplicon was 109, each individual had 1-8 MHC-I exon 3 alleles and in total 281 nucleotide sequences were uploaded to GenBank.

Parentage analysis

Parentage was analysed for 501 chicks. We used six fluorescently labelled microsatellites (Ase18, Ase25, Ase37, Ase48, Ase 58, Ppi2) in parents and offspring of 141 pairs, following methods in [26]. The PCR products were fragment analysed on a DNA sequencer ABI PRISM® 3100-AvantTM Genetic Analyser (Applied Biosystems) and the resulting chromatograms were analysed using ABI Genescan software. Exclusion probabilities were calculated with Cervus 3.0, and paternity analysis was conducted using the GeneMapper software (version 4.0). Extra-pair young were found in 14.6-32.7% of the nests across years.

150 In total 35 pairs had extra-pair young in their nest: 21 extra-pair males were identified (across
151 18 social pairs) and for 17 social pairs the extra-pair male could not be identified. There were
152 106 pairs with no extra-pair young in their nest.
153
154 *Calculating functional MHC-I distances as a measure of dissimilarity in pairs*
155 In order to assess MHC-dissimilarity (MHC-I functional distance) of all known reed warbler
156 pairs we estimated MHC-I functional distances according to [15]. We computed the MHC-I
157 functional distances as follows: The verified MHC-I sequences (N = 281) were translated
158 into a set of 243 unique amino acid sequences and from these we extracted the MHC-I exon 3
159 peptide-binding regions (PBR, inferred from [32]) that made up a set of 160 unique PBR
160 amino acid sequences. The PBR sequences were represented by five physicochemical
161 descriptors per amino acid [33], that were subsequently used to construct a maximum
162 likelihood tree with contml in the PHYLIP package, v 3.69. We used this tree (Fig. S1), as a
163 reference from which the overall MHC-I functional distances between pairs (considering the
164 full MHC-I repertoire of each male and female) were extracted with the UniFrac software
165 [34]. MHC-I functional distances were compiled for:; (1) *social pairs with extra-pair young*:
166 **SP-EPY** (N = 35 pairs), (2) *Extra genetic pairs*: **EGP**, the corresponding extra-pair dyads,
167 when known, to the SP-EPY social pairs (N=21 dyads corresponding to 18 of the SP-EPY as
168 a result of some males being the EGP for more than one SP-EPY), (3) potentially available
169 extra-pair males with a territory within a 60 m radius of SP-EPY nests where the EGP was
170 known, as all EGP males had a territory within 60 m of the SP-EPY nest (N=212 putative
171 males, range 6–14 per SP-EPY) and (4) *social pairs with only within-pair young:* **SP-WPY,**
172 i.e., pairs without EPY in the nest (N = 106 pairs). In total, MHC-I genotypes were available
173 for 128 females and 127 males in the population enabling MHC-I functional distances to be
174 calculated between 16256 theoretically possible pairs.

175

176 *Statistical analyses*

177 To test whether there was evidence of non-random pairing regarding MHC dissimilarity for
178 the different pair types (SP-WPY, SP-EPY of EGP) the position of the mean MHC
179 dissimilarity for each of the observed groups was compared against the distributions of mean
180 MHC dissimilarity from 10000 groups of random pairs (matched for the group size of the
181 observed means i.e.,106 for SP-WPY, 35 for SP-EPY and 21 for EGP). Random pairs were
182 sampled from all possible combinations of 128 females and 127 males genotyped in the
183 population (i.e., 16256 theoretically possible pairs) with replacement between (not within)
184 each of the 10000 iterations for each group size. If the observed mean MHC dissimilarity fell
185 outside the two-tailed 95% confidence intervals of the random distribution, then the observed
186 MHC dissimilarity was significantly different to that expected from random pairing given the
187 group size at the level of $P < 0.05$. To test whether females in SP-EPY choose extra-pair
188 males in a non-random fashion with regard to MHC dissimilarity, we generated 10000
189 random pairings between each female and all the surrounding males. Surrounding males were
190 males with a territory within 60 m of the nest. We then tested whether the MHC dissimilarity
191 of the observed EGP fell outside the two-tailed 95% confidence intervals of the distribution
192 of the random pairings. The randomisation analyses were conducted in R using base code.

193

194 Differences in the MHC-I dissimilarity scores between pair types (SP-WPY, SP-EPY of
195 EGP) were analysed with general linear mixed models (GLMM) using the R package lme4
196 version 1.1-29 [35]. The response variable was MHC-I dissimilarity scores, with pair type as
197 a three-level fixed effect. Female identity and year were included as random effects in the
198 models to account for the non-independence of pairings within the same year and the same
199 female with different male partners. The significance of the effect of pair type on MHC

dissimilarity was tested using a likelihood ratio test to compare the full and reduced model (*i.e.*, a model without pair type) using the Chi square distribution. To test whether MHC dissimilarity differed between SP-EPY and the EGP, the same model was run on a subset of the data that only included the paired results from SP-EPY where the EGP was known (18 SP-EPY and 21 EGP).

To examine the MHC dissimilarity of the SP-EPY and the EGP in the context of the putatively available extra-pair males, we used the MHC genotypes of the surrounding males. MHC dissimilarity scores were calculated between each focal female and all her putatively available extra-pair males (all males with a territory within a 60 m radius of the nest). This data was then used to calculate the percentile of the MHC dissimilarity of the SP-EPY and EGP within the distribution of the MHC dissimilarity offered by the surrounding males. For example, if the percentile of the SP-EPY was 0.2, then the MHC dissimilarity of the SP-EPY was lower than 80% of the potential pairings between the focal female and the other surrounding males. The difference between the percentile of the observed pairs MHC dissimilarity scores and that of the median (*i.e.*, 0.5) was tested using GLMMs. The response variable was MHC dissimilarity with the observed percentile (either SP-EPY or EGP) or median treated as a two-level fixed factor and pair included as a random effect.

All analyses were conducted in R version 4.1.1 [36]

Data availability

Genbank accession numbers are pending.

Results

225 MHC-I dissimilarity (functional distance) was calculated for three different pair types: social
226 pairs with extra-pair young (**SP-EPY**, N = 35 pairs); extra genetic pairs (**EGP**, N=21
227 occurrences) and social pairs with only within-pair young (**SP-WPY**, i.e. pairs without EPY
228 in the nest, N = 106 pairs). The mean MHC dissimilarity within each of the three pair types
229 was tested against what would be expected from random mating and the only group with
230 MHC dissimilarity scores outside what would be expected from random mating were the SP-
231 EPY, which had a significantly lower mean MHC dissimilarity score than would be expected
232 by chance from 35 pairs (Fig. 1).
233
234 The randomisation test suggests that SP-EPY are more similar than expected under random
235 mating. However, there was no overall significant difference in the MHC dissimilarity of the
236 three different pair types (SP-EPY, SP-EPY and EGP, Fig. 2, $\chi^2_{2, 158} = 3.75$, P = 0.15).
237 Though, there was a trend for SP-EPY to have lower MHC dissimilarity than the
238 corresponding EGP in the cases where the EGP could be identified (Fig. 2, $\chi^2_{1, 39} = 3.09$, P =
239 0.08). Moreover, after removing an outlying data point with extremely high MHC
240 dissimilarity from SP-EPY and its corresponding EGP, this difference became statistically
241 significant ($\chi^2_{1, 36} = 6.04$, P = 0.01).
242
243 Both the randomisation tests and the analyses comparing the pairwise MHC dissimilarity of
244 different pair types suggest that SP-EPY have lower MHC dissimilarity than other pair types.
245 To analyse this finding further, we compared the MHC dissimilarity of SP-EPY and the EGP
246 with all available extra-pair males within a 60 m radius of SP-EPY nests where the EGP was
247 known. The MHC dissimilarity of SP-EPY was significantly lower than the median MHC
248 dissimilarity of the female if paired with any of the other surrounding males (Fig. 3A, $\chi^2_{1, 37}$
249 = 5.06, P = 0.02), whereas no such difference was evident between EGP and the female if

250 paired with any of the other surrounding males (Fig. 3B, $\chi^2_{1, 37} = 2.03$, P = 0.15).
251 Furthermore, there was no evidence that females in SP-EPY selected the males in their EGP
252 in a non-random fashion, given the potential MHC dissimilarity represented by the
253 surrounding males (Fig. S2).
254
255 **Discussion**
256 Overall, there is evidence that female reed warblers in social pairs with low MHC
257 dissimilarity achieve higher MHC diversity in their chicks by engaging in extra-pair matings,
258 but that these extra-pair matings seem to occur at random with respect to MHC diversity.
259
260 We found that reed warblers pairs with extra-pair young in their clutches (social-pairs with
261 extra-pair young, SP-EPY) had significantly lower MHC-dissimilarity than expected from
262 random pairing. Furthermore, the MHC dissimilarity tended to be higher between females
263 and their extra-pair males (extra-genetic pairs, EGP) than between females and their SP-EPY
264 male. Therefore, high MHC similarity within social pairs is associated with paternity loss in
265 this population. Females in pairs with low MHC dissimilarity achieve improved MHC
266 compatibility through extra-pair mating, i.e., they obtained higher MHC dissimilarity, and
267 our data suggest that this could be achieved through random extra-pair mating with
268 surrounding territorial males.
269
270 The high frequency of nests with EPY in reed warblers (up to 33%) suggests that extra-pair
271 mating is very common in this population [25]. If most females engage in extra-pair matings,
272 then these copulations appear to result in extra-pair offspring more frequently when the MHC
273 dissimilarity of the social pair is low. In such a scenario, post-copulatory mechanisms (*e.g.,*
274 cryptic female choice or sperm competition) could select for successful fertilization by the

extra-pair male when the social male has a low degree of MHC dissimilarity with the female [37]. There is evidence for post-copulatory MHC-based mate choice in birds: an experimental study in domestic chickens showed that MHC-I dissimilarity predicted fertilisation success [38]. Moreover, MHC-dissimilarity has been shown to influence fertilisation success in Chinook salmon *Oncorhynchus tshawytscha* [1], mice [12,39] and even humans [40,41]. The observation in our reed warbler study that the EGP and the social-pairs with only within-pair young (SP-WPY) have random MHC dissimilarity whereas social pairs with extra-pair young (SY (SP-EPY) have lower MHC dissimilarity, supports the concept that MHC dissimilarity may play a role in fertilization success.

The pattern of higher MHC dissimilarity between EGP than SP-EPY raises the question of whether there is active choice for MHC dissimilar males for extra-pair copulations. However, comparisons between the MHC dissimilarity of EGP and the focal female with the surrounding putatively available males suggests this is not the case in our study of reed warblers. There was no significant evidence that the MHC dissimilarity of the EGP was in the upper range of the MHC dissimilarity that could potentially have been achieved by the female if she copulated with a different locally available male. Furthermore, there was no evidence of non-random pairing with the EGP with respect to the MHC dissimilarity of surrounding males. A lack of active choice for MHC dissimilar males when engaging in extra-pair copulations is consistent with our observation that extra-pair mating is extremely time-constrained in reed warblers, since the social male mate-guards the female (Halupka et al, unpubl.). Extra-pair mating may therefore be somewhat opportunistic and lack discerning choice. Indeed, given that the MHC dissimilarity of the SP-EPY was significantly below the median MHC dissimilarity of the focal female with other surrounding territorial males, it is likely that most locally available males represent an improved match in terms of MHC

dissimilarity compared to the social male. Therefore, even extra-pair mating at random is likely to result in an improved match in terms of MHC dissimilarity, which could potentially improve the MHC repertoire of the female's offspring.

Given that extra-pair matings in reed warblers are common, our results raise the intriguing possibility that reed warbler females who are in a social pair with poor MHC compatibility improve the MHC repertoire of her offspring through random extra-pair mating. Whilst previous work has suggested that extra-pair mating may be a mechanism by which the MHC repertoire of offspring is improved in passerine birds [14,27,28], ours is the first study, to our knowledge, to demonstrate that this could effectively be achieved through extra-pair mating occurring at random. When MHC compatibility with the social mate is poor, it may be the case that any other male represents a better genetic prospect to the female.

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Author contributions

LH designed the project, performed the fieldwork and MHC lab analyses, prepared the data for the statistical analyses, and contributed to the interpretation of the data. EO performed the statistical analyses and contributed to the interpretation of the data. MS performed the functional analyses of the MHC data and contributed to the interpretation of the data. HS performed fieldwork and microsatellite analyses. EK performed the fieldwork. DH contributed to the interpretation of the data. HW helped to plan the project, supervised the project, contributed to the statistical analyses and to the interpretation of the data, all co-authors contributed to writing of the article.

Competing financial interests

The authors declare that they have no financial competing interests.

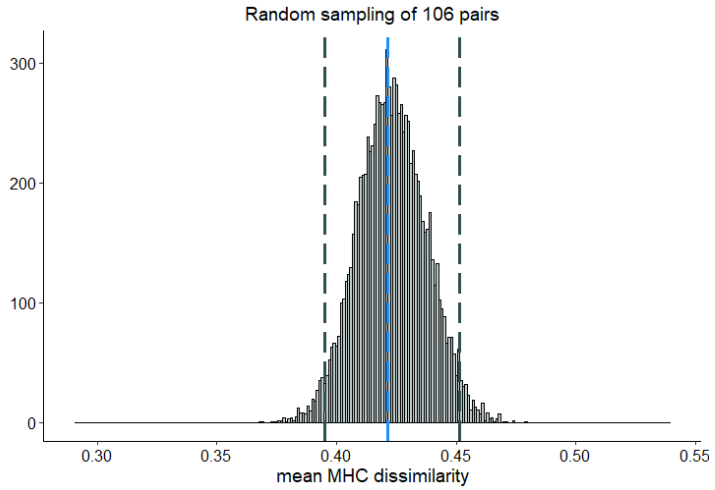
Ethics approval and consent to participate

The permits to conduct this study were issued by the Lower Silesian Regional Directorate of Nature Protection located in Wrocław, Poland (RDOŚ-02-WPN-6630/69/10/mr; WPN.6205.55/2011.MR; WPN.6205.39/2012.MR; WPN.6205.38.2013.MR2), II Ethical Committee in Wrocław, Poland (25/2006, 45/2009), and the Polish Ministry of Environment (DLOPiK-op/ogiz/III-19/4478/06/aj) and the Ornithological Station of the Polish Academy of Sciences, based on the decisions of the Polish Ministry of Environment (ringing licence

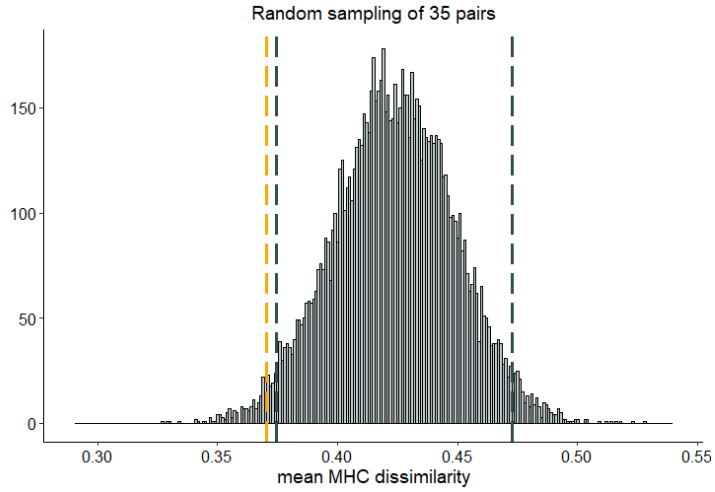
numbers: 78/2006, 83/2007, 87/2008, 93/2009, 95/2010, 98/2011, 103/2012).

Figures

a)


Random sampling of 106 pairs
300
200
100
0
0.30
0.35
0.40
0.45
0.50
0.55
mean MHC dissimilarity

b)


Random sampling of 35 pairs
150
100
50
0
0.30
0.35
0.40
0.45
0.50
0.55
mean MHC dissimilarity

c)

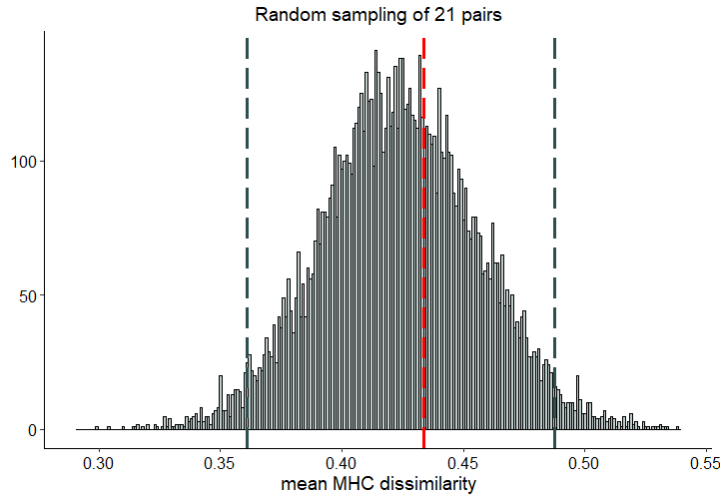
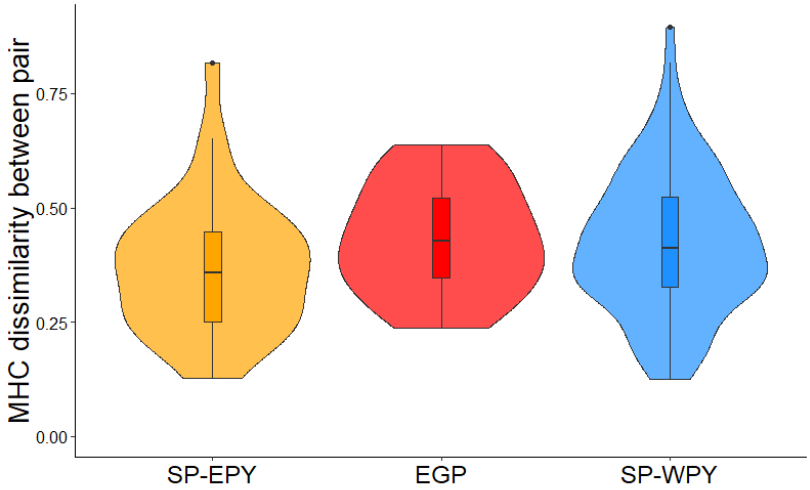

Random sampling of 21 pairs
100
50
0
0.30
0.35
0.40
0.45
0.50
0.55
mean MHC dissimilarity

Figure 1: Distribution of 10000 mean MHC dissimilarity scores calculated from randomly selected groups of pairs using all MHC genotyped individuals in the population (128 females and 127 males). Group sizes were matched for the sample size of the comparison dataset: (**a**) 106 pairs for the social pairs with only within-pair young SP-WPY, (**b**) 35 pairs for the social pairs with extra-pair young SP-EPY and (**c**) 21 pairs for extra genetic pairs EGP. Dark grey dashed lines depict the lower 2.5% and upper 97.5% of the data in both figures (95% confidence intervals). The mean MHC dissimilarity for the SP-WPY, SP-EPY and EGP are depicted by the blue, orange and red dashed lines, respectively.

a)



b)

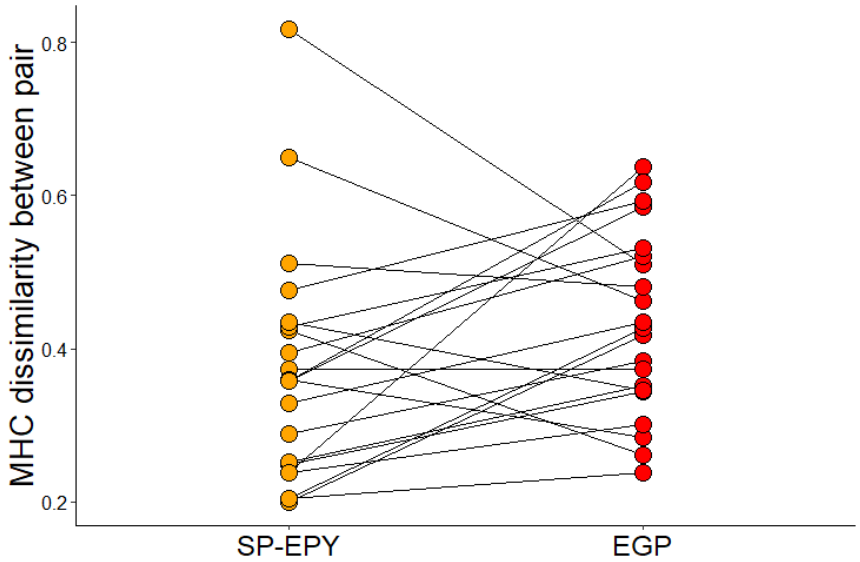
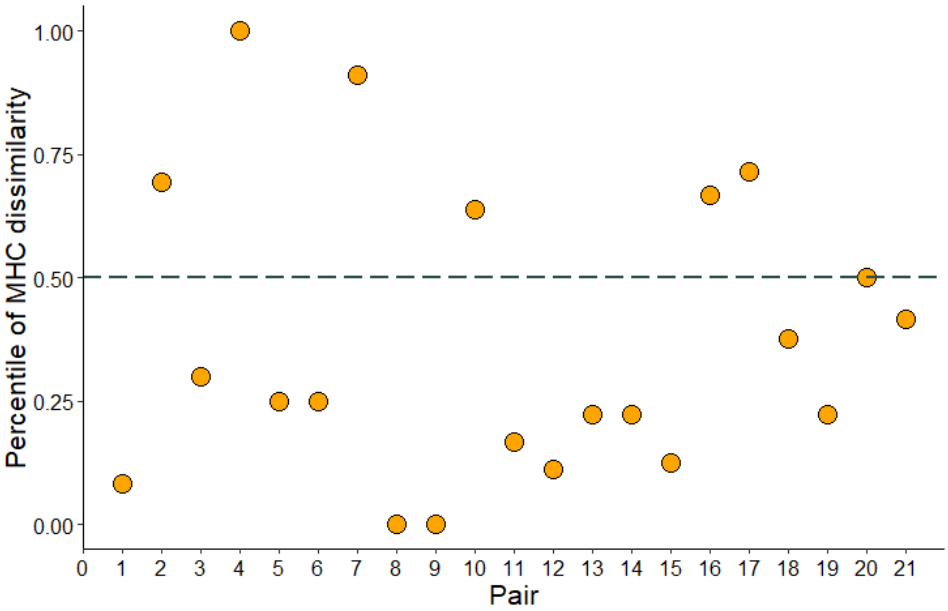


Figure 2: MHC dissimilarity scores between different pair types. **a)** Violin plot of the MHC dissimilarity between social pairs with extra-pair young (SP-EPY, n = 35), females with extra-pair young and their known extra-pair male (extra genetic pair, EGP, n = 21) and social pairs with only within-pair young in their nest (SP-WPY, n = 106). Boxplots within the violins (central line = median, whiskers = 1.5 times the interquartile range of the data, black dots = outliers). **b)** Comparison of the MHC dissimilarity between SP-EPY where the extra-pair male is known (n = 18) and known EGPs (n = 21). Lines link the female with the corresponding extra-pair males.

a)



b)

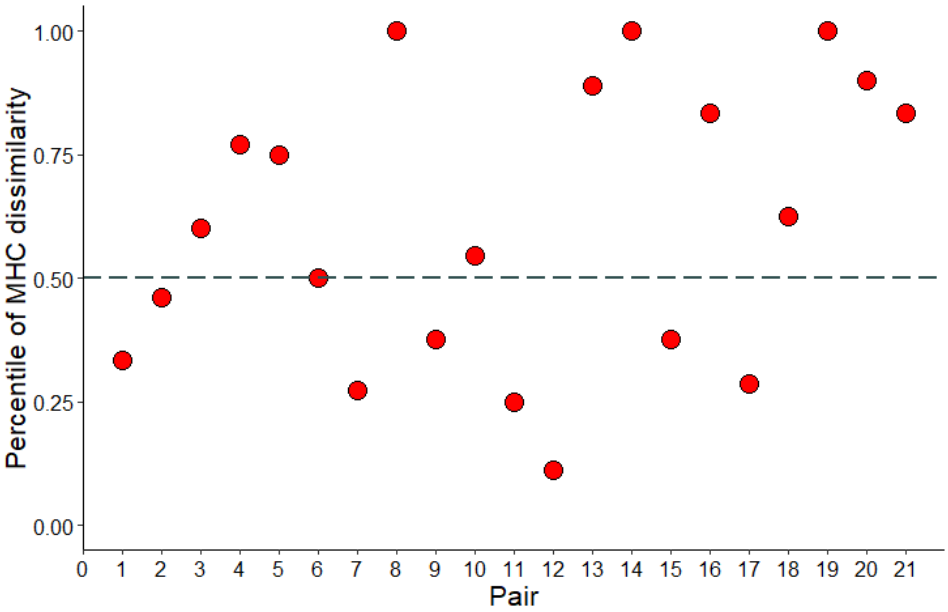


Figure 3: The percentile of the MHC dissimilarity of (**a**) social pairs with extra-pair young SP-EPY and (**b**) extra genetic pairs EGP within the distribution of MHC dissimilarity between the focal female and all potentially available extra-pair males within a 60m radius of her nest. Dark grey dashed line denotes the median.