Social and extra-pair mating in relation to major histocompatibility complex variation in common yellowthroats

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Females are thought to gain better-quality genes for their offspring by mating with particular males. Genes of the major histocompatibility complex (MHC) play a critical role in adaptive immunity, and several studies have examined female mate choice in relation to MHC variation. In common yellowthroats, females prefer males that have larger black facial masks, an ornament associated with MHC variation, immune function and condition. Here we also tested whether mating patterns are directly correlated with MHC diversity or similarity. Using pyrosequencing, we found that the presence of extra-pair young in the brood was not related to male MHC diversity or similarity between the female and her within-pair mate. Furthermore, extra-pair sires did not differ in overall diversity from males they cuckolded, or in their similarity to the female. MHC diversity is extremely high in this species, and it may limit the ability of females to assess MHC variation in males. Thus, mating may be based on ornaments, such as mask size, which are better indicators of overall male health and genetic quality.

Keywords: good genes; compatibility; immunity; extra-pair mating; warbler

1. INTRODUCTION

An increasing number of studies suggest that female mate choice is influenced by the genetic characteristics of potential mates (reviewed by [1–3]). In particular, studies have focused on two main types of genetic benefits to females: ‘good genes’ and ‘compatible genes’. Good-genes benefits occur when particular alleles increase offspring fitness independent of the rest of the genome, and thus all females benefit from mating with males that possess good alleles [2]. Fitness benefits from compatible genes are caused by the interaction of genes, so a male’s suitability to a female will be dependent upon both of their genotypes. Compatible-genes effects can occur several ways, including female preferences for mates that are dissimilar [4,5] or relatively heterozygous in general (e.g. inbreeding avoidance [6]), or at particular loci, such as the major histocompatibility complex (MHC). Studies of the MHC provide some of the best examples of good-genes and compatible-genes effects, as well as illustrating how these effects can overlap [7–9].

In vertebrates, genes of the MHC code for molecules that bind to foreign peptides and present them to T cells, thus initiating the adaptive immune response [10]. A number of studies have demonstrated associations between specific alleles and pathogen resistance [11,12], so females choosing a mate having a ‘good’ MHC allele may increase the disease resistance of their offspring. Consistent with this idea, female great snipes (Gallinago media [13]) and fat-tailed dwarf lemurs (Cheirogaleus medius [9]) preferred males with particular genotypes, and in sticklebacks (Gasterosteus aculeatus [7]) males with the preferred genotype were also more resistant to parasites. The MHC is highly polymorphic in most populations, and selection from pathogens is also thought to favour individuals heterozygous at these genes, because they may be able to present a wider range of foreign peptides to T cells [14].

Thus, females could benefit from mating with males that have high MHC heterozygosity or diversity, because their offspring are likely to be heterozygous and resistant to pathogens. In support of this hypothesis, several studies have found that females preferred males with a relatively greater number of MHC alleles [9,15], or that social mates with low MHC diversity were more likely to be cuckolded [16,17].

Females could also choose mates that are more compatible with respect to the female’s own MHC genotype, resulting in a beneficial combination of MHC alleles in the offspring. The most common pattern appears to be a bias towards matings between females and males that are relatively MHC-dissimilar, which occurs in mammals [18,19], fish [20,21], reptiles [22,23] and birds [24,25]. This disassortative mating at the MHC could reduce inbreeding and increase the heterozygosity of the offspring. Alternatively, some evidence suggests that females may prefer mates with similar MHC genotypes [15,26,27]. A preference for similar mates may avoid the disruption of co-adapted gene complexes or production of offspring with too many MHC alleles, which could result in reduced immunocompetence [28,29].

Lastly, a few studies show that fitness traits are optimal in individuals with an intermediate number of alleles [30–32], and females prefer mates with an intermediate
similarity to themselves in trout (Salmo trutta [33]) and sticklebacks [7]. However, it should be noted that in some species there is only weak (tuataras, Sphenodon punctatus [22]) or no evidence of MHC-dissimilar mating (Soay sheep, Ovis aries [34]; great reed warblers, Acrocephalus arundinaceus [35]).

We studied the relationship between MHC genotype and mate choice in the common yellowthroat (Geothlypis trichas), a sexually dimorphic warbler. Common yellowthroats are socially monogamous, but 46 per cent of nests have extra-pair young (EPY) [36]. Males have two prominent plumage ornaments: a black facial mask, and a yellow-coloured throat and breast (bib); females lack the mask and have a more subdued bib. In our Wisconsin study population, sexual selection (from both social and extra-pair mating) favours males with larger black masks, but there is no evidence of selection on the bib [36]. Mask size might be an indicator of good-genes benefits, as males with larger masks weigh more and have higher IgG antibody levels [37, 38], and under some environmental conditions, EPY exhibit stronger T-cell-mediated immune responses than within-pair nest-mates [39]. Recently, we found that male mask size and apparent survival are both positively associated with the total number of MHC class II alleles, and susceptibility to malaria infection is associated with the presence of a particular class II allele [40].

Thus, preference for a male ornament may confer indirect benefits in the form of good genes to female common yellowthroats; however, females could also be choosing mates based on MHC compatibility. In this study, we compared MHC genotypes of females with their social and extra-pair mates to determine whether within-pair or extra-pair matings were related to overall MHC diversity of males or the compatibility of female and male genotypes. In terms of MHC diversity, we expected females to choose males that had more alleles, as this is likely to be associated with heterozygosity. In terms of similarity (or compatibility), we expected the male mated to a female to possess a relatively lower degree of MHC similarity to the female than males that were not mated to her. We tested these predictions using 454 pyrosequencing, because common yellowthroats have an unusually high number of MHC gene duplications compared with other species [41]. Recent studies indicate that pyrosequencing provides more accurate estimates of MHC variation than older methods for species with high gene copy number [42, 43]. We genotyped birds at both MHC class I and II loci, which are involved in presenting peptides derived from intra- and extra-cellular pathogens, respectively. Thus, our analysis of both MHC class I and II genes in social and extra-pair mates provides one of the most comprehensive studies to date of MHC-based mate choice in a wild songbird.

2. METHODS

(a) Field procedures

Common yellowthroats were studied at the University of Wisconsin—Milwaukee Field Station in Saukville, Wisconsin during 2002–2005 [38, 39]. Individuals (adults and nestlings) were colour-banded for individual identification, and censused at least three times per week to determine arrival date. At banding, plumage ornaments were measured [36], and blood samples were taken for molecular analyses of paternity and relatedness [39]. Paternity of nestlings was assessed, and the sires of EPY were identified using four microsatellite loci [39].

(b) Determining major histocompatibility complex genotypes

Forty-two males and 36 females were screened at both class I and II loci, targeting the exons encoding the peptide-binding regions (PBRs) of the MHC molecules (see the electronic supplementary material for more details). As in other passerines, MHC genes in the common yellowthroat are highly duplicated [41] and our primers amplify multiple loci at once. In this study, a single individual could have up to 18 class I and 46 class II sequences, which implies at least 9 and 23 loci, respectively. As in previous studies, we call these different sequences ‘alleles’ for simplicity, but recognize that they come from different loci. Our methods for filtering sequences to determine the number of alleles in an individual have been described previously [40] and are also presented in the electronic supplementary material. Three individuals (one male and two females) sequenced poorly at class II, so only their class I genotypes were included in further analyses. Repeatability of the total number of alleles was high ($R = 0.92, F_{R} = 22.76, p < 0.001$) using independent PCRs in two separate pyrosequencing runs (eight birds tested at class I and one at class II).

In these two runs, we found 87 per cent (76/87 alleles) of all class I alleles both times in the same individual. At class II, we found 86 per cent (18/21) of alleles in both runs. Sequences of all alleles are available in GenBank (accession nos GQ247571, GQ247577, GQ247583–GQ247584, GQ247491, GQ247593, GQ247598, GQ247601–GQ247602, GQ247605, GQ247611– GQ247612, GQ247615, GQ247618, GQ247620–GQ247626, GQ247629, GQ247633, GQ247635–GQ247636; JQ859960–JQ859963; JK213814–JK215140). Lastly, we identified sequences that appeared to be non-functional, based on stop codons and frameshift mutations, as well as sequences that did not appear to be transcribed, based on analysis of cDNA. These non-functional sequences were excluded from further analyses (see the electronic supplementary material).

(c) Major histocompatibility complex diversity of mates

We examined MHC diversity of social and extra-pair mates relative to males that were apparently less preferred (unpaired males and males with EPY in their nests, respectively). Our estimates of MHC diversity included: the total number of alleles in an individual and the number of rare alleles. The total number of alleles within an individual excluded non-functional alleles and alleles with duplicate amino acid sequences. If MHC diversity is maintained by cycles of host–parasite evolution, then based on negative frequency-dependent selection we might also expect rare alleles to be at a selective advantage [44]. In this case, there might be assortative mating based on the number of rare alleles in an individual (e.g. females would prefer males with more rare alleles). We estimated the number of rare alleles as the number of alleles in an individual have been described previously [40] and are also presented in the electronic supplementary material. Three individuals (one male and two females) sequenced poorly at class II, so only their class I genotypes were included in further analyses. Repeatability of the total number of alleles was high ($R = 0.92, F_{R} = 22.76, p < 0.001$) using independent PCRs in two separate pyrosequencing runs (eight birds tested at class I and one at class II).

(d) Mate similarity

To assess compatibility of mates, we examined two measures of overall MHC similarity, as well as similarity at particular
MHC alleles. For our first estimate of overall similarity, we used GELSTATS v. 2.6 [45] to calculate allele-sharing values for each pair of individuals as twice the sum of the alleles shared by two individuals divided by the total number of alleles present in both individuals following Wetton et al. [46]. Allele sharing does not take into account functional similarity of individuals, so we also calculated a second estimate based on amino acids. We calculated the proportion of amino acid differences between mates for each allele (p-distance) in MEGA v. 5.05 [47], and then averaged those values for all the pairwise comparisons of alleles between the two individuals. We calculated this amino acid difference based on (i) the entire exon sequence and (ii) the putative PBRs, which should be under positive selection. Putative PBRs were identified for class I following Promerová et al. [48] and references therein, and for class II following Tong et al. [49]. As a neutral comparison, Queller & Goodnight’s [50] coefficient of relatedness was calculated using 10 microsatellite loci (for more details see [40]).

We also examined whether mated pairs showed non-random patterns of association at particular alleles. We focused on the two and three most common alleles at class I (GenBank accession nos JQ859962–JQ859963) and class II (GenBank accession nos GQ247621, JQ859960–JQ859961). These alleles were found in at least a third of males in our previous study [40], and it allowed us to examine particular alleles while limiting the number of statistical tests to a manageable number.

### Data analysis

We examined the MHC diversity of apparently preferred and less preferred mates (total number of alleles and number of rare alleles), as well as the similarity to females of these males (proportion of alleles shared, amino acid distance and the presence of common alleles) in four sets of comparisons. First, we used paired t-tests to compare the social mate with nearby ‘unpaired’ male(s) a female could have paired with but who were not chosen, and apparently were less preferred. These ‘unpaired’ males did not have a mate at the time the focal female settled, but most eventually gained a social mate. In cases where multiple unpaired males were present (up to three), values were averaged. Second, we used independent-sample t-tests to compare MHC diversity and similarity between nests with and without EPY, assuming that males with some EPY were less preferred than males without any EPY. Third, we used paired t-tests to examine MHC diversity and similarity for within-pair males and extra-pair sires in the same nest. Lastly, we used paired t-tests to compare the MHC diversity and similarity of known extra-pair mates with those of potential extra-pair mates (neighbouring males). Paternity analyses indicate that 80 per cent of EPY are sired by immediate neighbours [51]. Each female had between one and five neighbouring males, and MHC data for these males were averaged when there were multiple males. It is possible that females use MHC cues after using mask size as their primary cue, so to control for the possible effects of mask size, we also repeated these tests using mixed models with mask size as a fixed effect and nest identity as a random effect. However, the results of these tests were qualitatively similar to the paired comparisons (see the electronic supplementary material).

#### 3. RESULTS

Common yellowthroats exhibited a high degree of MHC variability. At the population level, we recovered 298 unique class I sequences from 78 individuals, and 977 class II sequences from 75 individuals. When non-functional alleles and alleles with duplicate amino acid sequences were excluded from genotypes, individuals had 8.8 ± 0.3 (range: 4–15) class I alleles and 27.7 ± 0.7 (range: 10–45) class II alleles. Most of the class I alleles were rare (n = 225), and individuals had an average of 4.9 ± 1 (range: 1–8) rare alleles. At class II, 699 alleles were rare, and individuals had 13 ± 1 (range: 2–22) rare alleles. The number of alleles was not biased by read number as there was no correlation between the number of alleles in an individual and number of reads at class I (r² = 0.02, n = 78, p = 0.21). There was a weak positive correlation at class II (r² = 0.07, n = 75, p = 0.03), but not when two outliers were removed (r² = 0.03, n = 73, p = 0.12).

#### (a) Social mates

In general, social pairing was not related to MHC variation at either class I or II. First, we compared the MHC of within-pair mates with that of nearby unpaired males. At class I, the total number of alleles was lower for within-pair mates (mean of 8.7 alleles) than unpaired males (12 alleles, p = 0.0003; table 1), even after...
was not related to the number of class I alleles of male diversity total no. alleles in male I 8.429 \pm 0.580 (21) 8.500 \pm 0.564 (20) 0.088 0.930 class II alleles (later-arriving females have within-pair mates with fewer so we tested for an effect of arrival date and found that the breeding grounds might limit their choice of mates, correcting for multiple tests (critical \( p = 0.005 \) based on Bonferroni correction for 10 tests). However, within-pair mates and unpaired males did not differ in the total number of class II alleles, nor the number of rare class I or II alleles (table 1). The date when females arrive on the breeding grounds might limit their choice of mates, so we tested for an effect of arrival date and found that later-arriving females have within-pair mates with fewer class II alleles (\( r^2 = 0.16, F_{1,31} = 5.76, p = 0.023 \)). When females arrived later in the season they also had to choose among unpaired males with smaller masks (\( r^2 = 0.17, F_{1,25} = 5.26, p = 0.031 \)). Female arrival date was not related to the number of class I alleles of within-pair mates (\( r^2 = 0.04, F_{1,32} = 1.21, p = 0.28, \) but later-arriving females had potential extra-pair mates with fewer class I alleles (\( r^2 = 0.34, F_{1,13} = 1.21, p = 0.022 \)). This relationship was driven by two influential data points, so it needs further study.

In terms of relative similarity between females and their mates, within-pair mates and unpaired males did not differ in the number of MHC alleles they shared with the female, nor did they differ in their amino acid distances to the female (table 1). We also found that social mates did not pair assortatively or disassortatively with respect to the common alleles at class I (contingency tests: \( n = 41, \text{both } p > 0.7 \)) or class II (\( n = 38, \text{all } p > 0.1 \)). Finally, similarity to the female did not differ between within-pair mates and the unpaired males at microsatellite loci (table 1).

### (b) Occurrence of extra-pair paternity

The presence of EPY in a brood was not related to MHC diversity or similarity. The total number of MHC alleles and the number of rare alleles possessed by the within-pair mate did not differ between broods with and without EPY (table 2). Similarity to the female was also not related to the presence of EPY (table 2). Furthermore, the presence of EPY in a brood was not related to whether males had the five most common alleles at either class I (contingency tests: \( n = 41, \text{both } p > 0.5 \)) or class II (\( n = 40, \text{all } p > 0.3 \)). Interestingly, pairs with a higher proportion of EPY in their brood were more dissimilar at class II (greater amino acid distances; \( r^2 = 0.170, n = 38 \text{ pairs, } t = 2.71, p = 0.01 \), without correction for multiple tests; figure 1), but not at class I loci (\( r^2 = 0.032, n = 41, t = 1.13, p = 0.26 \)).

### (c) Extra-pair mates versus within-pair mates

Extra-pair sires did not differ from the males they cuckolded in MHC diversity or similarity to the female (table 3). Neither the total number of MHC alleles nor the number of rare alleles per individual differed between extra-pair and within-pair mates. Likewise, the proportion of alleles shared with the female, the amino acid distances from the female and the similarity at microsatellite loci did not differ between extra-pair and within-pair mates (table 3). Finally, within-pair and extra-pair mates had similar frequencies of the common alleles at both class I and II (contingency tests: \( n = 19, \text{all } p > 0.2 \)).

### (d) Extra-pair sires

Males that gained extra-pair paternity did not differ from other neighbouring males (potential extra-pair sires) in MHC diversity or similarity to the female (table 4). Compared with neighbouring males, extra-pair sires had similar numbers of total class I and II alleles, similar
numbers of alleles shared with the female, and similar amino acid distances from the female. Similarity to the female at microsatellite loci also did not differ between extra-pair mates and neighbouring males (table 4).

4. DISCUSSION

By mating with preferred males, females may gain indirect benefits for their offspring, such as good genes, compatible genes or increased heterozygosity. Previous studies of mating in relation to MHC variation have found that mates often have higher MHC diversity, possess certain alleles or exhibit a non-random degree of MHC similarity to the female (reviewed by Huchard et al. [53]). In the common yellowthroat, we found little evidence for a relationship between MHC variation and mating with either within-pair male or extra-pair male, despite extensive sampling at both MHC class I and II. In general, within-pair mates did not differ from neighbouring males in their MHC diversity or their similarity to the female, and extra-pair mates did not differ from the within-pairs mates they cuckolded. We found two interesting relationships: (i) within-pair mates had lower variation at class I loci than nearby unpaired males (table 1), and (ii) there was a greater proportion of EPY when the female was more dissimilar to her within-pair mate (figure 1). These patterns were unexpected, but they are consistent with mate choice for intermediate levels of MHC variation and the maintenance of co-adapted gene complexes. Overall, however, there was little strong evidence that within-pair or extra-pair mating were related directly to MHC variation. Instead, we suggest that females are most probably to gain indirect benefits primarily through their preference for a male plumage ornament.

(a) Absence of mating based directly on major histocompatibility complex variation

Female yellowthroats prefer within-pair and extra-pair males with larger black facial masks [36,51,54], and mask size is positively related to male MHC diversity and survival (number of class II alleles; [40]). Thus, we expected females to mate with social and extra-pair males that had relatively more MHC alleles, and possibly more rare or dissimilar alleles as well. The lack of association between MHC variation and mating may be due to several constraints. One of the most important constraints is that mask size is not a perfect predictor of MHC variation. Although mask size was positively related to the number of class II alleles in males (n = 42 males, p < 0.001), it explained only 21 per cent of the variation in the number of class II alleles after controlling for age effects [40]. If females are choosing mates based on mask size, and not more direct cues from the MHC (e.g. odour), then mask

Table 3. Paired comparisons of MHC and microsatellite variation between within-pair and extra-pair mates. Comparisons were made between within-pair and extra-pair mates (male diversity), and between the similarity estimates for the female and each type of male (similarity to the female). Means are given ± s.e. t- and p-values are from paired t-tests. See §2 for details of each estimate.

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<th>extra-pair sires</th>
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<th>p-value</th>
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Table 4. Paired comparisons of MHC and microsatellite variation between extra-pair sires and potential extra-pair males. Comparisons were made between extra-pair and potential extra-pair (neighbouring) males (male diversity), and between the similarity estimates for the female and each type of male (similarity to the female). Means are given ± s.e. See §2 for details of each estimate.

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size provides a limited amount of information about MHC variation. Furthermore, even though MHC variation is important to immunity and survival, it is just one component of male fitness. In common yellowthroats, males with larger masks are older, weigh more and have greater antibody levels [36,37], and thus it is possible that mask size is a better indicator of overall male health and genetic quality than MHC variation per se. As pointed out by Hamilton & Zuk [55], if females are searching for healthy mates with genes for resistance to current pathogens and the expression of their ornaments is related to health, then females should base their mate choice on those ornaments (see also [56]).

A second constraint may be the ability of females to choose mates based directly on characteristics of the MHC. Although we have evidence that females discriminate among males based on mask size, it is still possible that they also use some more direct cues about MHC variation, such as differences in odour, which have been found in mammals and fish [56]. Evidence is starting to accumulate that birds can discriminate between individuals based on odour cues from feathers and preen gland secretions [57,58], and that odours are related to heterozygosity at microsatellite markers [59]. However, mate choice based on MHC-specific odours may be further complicated by the extensive diversity of the MHC in common yellowthroats, both in the number of alleles per individual, and the number of alleles in the population. This diversity renders almost all individuals MHC-dissimilar (only 6–9% shared alleles; table 1). This variability may make it difficult for females to assess their relative similarity to different males. In addition, odour cues can only indicate a particular MHC genotype, not whether that particular genotype is especially fit in a given environment, so a simpler method to choose disease-resistant mates may be based on ornament expression [56].

A third constraint on MHC-based mating is the confounding effect of male–male competition. For example, in the tuatara Miller et al. [22] found a weak pattern of MHC-dissimilar mating; however, male body size was a much stronger predictor of mating success, suggesting male–male competition outweighed MHC-based female choice in determining matings. Similarly, male–male competition may be the primary determinant of paternity in Soay sheep, which also showed no evidence of MHC-associated mating preferences [34]. In common yellowthroats, males arrive on the breeding grounds prior to females and compete with other males for territories. Thus, female choice of within-pair mate is first limited to males that gain breeding territories, and then further constrained by the availability of unpaired males [36]. As might be expected, we found that later-arriving females were paired with males that had smaller masks and fewer class II alleles, which we have found are associated with mask size and survival [40]. Furthermore, the territory that a female settles on could limit, in turn, the pool of potential extra-pair mates, as extra-pair sires are usually neighbouring males [36,51]. We found some support for this idea in terms of the number of class I alleles, but our sample size was small (n = 15) and the relationship depended on two influential data points. Thus, both male–male competition and female arrival date may weaken associations between mating and MHC variation in territorial migratory species such as the common yellowthroat.

A handful of other studies have found associations between mating behaviour and MHC variation in birds, and there is support for mating based on both diversity and similarity in different species. For example, extra-pair paternity was more common in Savannah sparrows (Passerines melanispinis) when the female and her within-pair mate were more similar at the MHC [24]. In rosefinches (Carpodacus erythrinus) [16] and Seychelles warblers (Acrocephalus seychellensis) [17], however, extra-pair paternity was more likely when within-pair males had lower class I diversity, and there was no effect of similarity on mating patterns. Although matings between dissimilar individuals are often predicted, there is also some evidence in house sparrows [15] and tiger salamanders (Ambystoma tigrinum) [26] for positive assortative mating at the MHC, similar to our results in which extra-pair paternity was lower in pairs that were more similar at class II (figure 1). One explanation for these results is that females are choosing mates with an intermediate level of MHC diversity to avoid reduced immunocompetence [28]. However, in common yellowthroats, there was little evidence that the level of extra-pair paternity was lowest at intermediate levels of MHC similarity (the relationship was relatively linear in figure 1). Another possibility is that females choose similar mates to avoid disruption of locally adapted gene complexes [60], but this remains to be studied in more detail.

(b) Conclusions

Females can gain genetic benefits via multiple avenues, and may assess cues for good genes through male traits, such as ornaments, genetic variation or both simultaneously. In common yellowthroats, neither within-pair nor extra-pair matings were related to characteristics of the MHC. This may be due to the extreme MHC diversity among individuals, which may influence the ability of females to assess MHC variation in males. In addition, both within-pair and extra-pair mating may be constrained by male–male competition and female arrival date. Thus, mating based on a plumage ornament, such as male mask size, may be a better integrator of overall male health and genetic quality, even if it is an imperfect predictor of MHC variation.

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