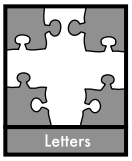

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Occurrence of extra-pair paternity is connected to social male's MHC-variability in the scarlet rosefinch *Carpodacus erythrinus*

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Genes of the Major Histocompatibility Complex (MHC) represent an essential component of the vertebrate acquired immune system. In the last decades, the role of MHC genes in mate choice has been subject of particular scientific interest. However, results of studies dealing with this topic in different species are equivocal and mechanisms conducting MHC-based mate choice are still puzzling. We investigated the impact of MHC class I variability on within-pair and extra-pair fertilisation success in a wild population of a socially monogamous passerine bird with considerable rates of extra-pair paternity, the scarlet rosefinch *Carpodacus erythrinus*. We found some support for the 'good-genes-as-heterozygosity model', as social males of high MHC-heterozygosity were cheated by their females less frequently than less MHC-heterozygous males. However, cuckolding males were not more MHC-heterozygous than the cheated social males, nor were extra-pair young more MHC-heterozygous than within-pair young. We did not find any evidence for mating preferences according to the complementarity model.

In recent years, the tools for studying genetic aspects of mate choice have improved significantly (Mays and Hill 2004). It has been proved that females may optimize their choice not only by choosing males exhibiting the most elaborate ornaments (the 'good genes' model, Mays and Hill 2004) but also according to their own genotype by disassortative mating, which is referred to as the genetic complementarity model (also known as compatibility, see e.g. Pialek and Albrecht 2005, Mays et al. 2008). According to the 'good genes' model, males with certain phenotypic traits should be generally preferred by females in a population as they might confer advantageous alleles increasing offspring quality (e.g. Iwasa et al. 1991), assuming that there is additive genetic variation in fitness. According to the complementarity model female preferences depend on their own genotype and they aim at the best possible combination of maternal and paternal genes to create optimal offspring genomes gaining non-additive genetic benefits for their progeny (reviewed in Hettyey et al. 2010). However, evidence shows that in some species the pattern is not unequivocal, but mate choice might be a complex of both of these (Roberts and Gosling 2003). As reviewed in Hettyey et al. (2010) there is a lack of studies examining mate choice (mainly extra-pair mating) in the frame of both 'good genes' and 'complementarity' models in relation to particular genes.

Via their choosiness females may obtain direct benefits, e.g. male's territory, nuptial food gifts or male's ability to fertilize ova, or indirect benefits, i.e. genes that confer increased offspring viability (Andersson 1994). In some mating systems, for example in lekking birds or in socially monogamous avian species where females engage in extra-pair copulations (EPC), exclusively indirect benefits are obtained by females since all that males or extra-pair males, respectively, contribute to the offspring are genes (reviewed in Griffith et al. 2002).

From the perspective of evolutionary biology, extra-pair mating offers a particularly useful model for the investigation of the indirect benefits rising from mating with males differing in quality. Extra-pair mating has been recorded in approximately 90% of avian species and if we take into account only the socially monogamous species (in which extra-pair paternity [EPP] is twice as common as in the polygynous species, Hasselquist and Sherman 2001) then the level of EPP is estimated on average to 11% of offspring and approximately 19% of broods (Griffith et al. 2002). Thus far, we do not understand EPPs enough to fully comprehend the EPC behaviour. For instance, as proposed by Arnqvist and Kirkpatrick (2005) EPCs may represent a solely male offensive strategy bringing no sufficient benefits to females. However, this conclusion was exposed to severe criticism (Griffith 2007), and as there is evidence suggesting that females may directly search for EPCs and initiate them (Kempenaers et al. 1992, Birkhead and Møller 1993, Strohbach et al. 1998, Bouwman et al. 2006, Dunn and Whittingham 2007), the question concerning female

benefits from EPFs remain unresolved. In socially monogamous species EPCs might be the only way how females could get offspring with chosen males when these are already paired (Westneat et al. 1990, Birkhead and Møller 1993) or when their previous social-partner choice showed to be inappropriate (reviewed in Jennions and Petrie 2000), but we clearly need more evidence concerning these potential indirect benefits of extra-pair fertilisations (EPFs) to females.

Genes of the major histocompatibility complex (MHC) play a crucial role in the vertebrate acquired immunity (Klein 1986). They encode glycoproteins which bind antigen peptides and present them on cell surfaces to T cells. If the antigen peptide is recognised by a T cell, an immune response is triggered (Abbas et al. 1994). The MHC genes are under strong positive selection, acting mainly on amino-acid sites involved in antigen binding (peptide-binding region). In the last decades they have been subject of particular interest as they were shown to influence mate choice in several non-model species of mammals (Schwensow et al. 2008), fish (Eizaguirre et al. 2009), amphibians (Bos et al. 2009), reptiles (Miller et al. 2009), and also in birds (Richardson et al. 2005, Bonneaud et al. 2006).

Evidence shows that resistance to a specific parasite is ensured mostly by one or only few MHC alleles (e.g. Bonneaud et al. 2005, Loiseau et al. 2008, Mankowski et al. 2008, Fraser and Neff 2010; reviewed in Jeffery and Bangham 2000). Overdominance hypothesis (reviewed in Piertney and Oliver 2006) assumes that the more MHC alleles an individual has, the higher should be its resistance to a wide spectrum of pathogens. It might be difficult to distinguish which alleles are advantageous, therefore it has been suggested that as 'the best of a bad job' it might be convenient for individuals to mate with MHC-dissimilar mates to produce the most MHC-heterozygous offspring (Milinski 2006, reviewed in Piertney and Oliver 2006). Also mating with highly MHC-heterozygous mates might be beneficial, because it has been shown that highly MHC-heterozygous parents produce highly MHC-diverse young (Bonneaud et al. 2006). On the contrary, theoretical models (Nowak et al. 1992) suggest that too high heterozygosity on MHC might be disadvantageous. This is because of increased loss of T-cell variability due to negative selection of autoreactive T-cell clones in thymus. When there are too many MHC molecules, too many peptide variants are generated from self proteins leaving less peptide variants to be recognised as non-self by the T cells. Therefore it was suggested that an individual should possess an optimal rather than maximal number of MHC alleles (Milinski 2006, Woelfing et al. 2009). This was evidenced by some experimental studies (Hill et al. 1991, Ilmonen et al. 2007, Bos et al. 2009). If individuals optimize mate-choice to achieve an optimal level of MHC-heterozygosity, rather than maximal, then mates of an intermediate level of MHC-dissimilarity should be preferred (Milinski 2006, Eizaguirre et al. 2009). Here, we evaluate the hypotheses of 'good genes as heterozygosity' and complementarity in a mating system of social monogamy with considerable rates of extra-pair fertilisations, studying MHC class I diversity. We examined the variation of exon 3, which encodes parts of the peptide-binding region. Our model species, the Scarlet rosefinch, is a sexually dichromatic long-distance migratory passerine with delayed plumage maturation and high levels of plumage ornament variability in males (Stjernberg 1979). It is a socially monogamous species with moderate rates of extra-pair paternity (almost 40% of nests contained extra-pair young; Albrecht et al. 2007). It breeds once a year and the breeding season is extremely short (Björklund 1990). Females build social pairs with males immediately after arrival on the breeding site, but later some of them have young also with males outside the pair-bond (Albrecht et al. 2007).

Materials and methods

Study population and field procedures

We studied a population of Scarlet rosefinches nesting in the Šumava Mountains National Park, Czech Republic (48°49′ N, 13°56′ E, ~750 m a.s.l.). A detailed description of the study site and field procedures is given in Albrecht et al. (2007). The dataset included samples of 614 individuals (108 nests, 70 females, 91 males and 453 nestlings) collected during breeding seasons of 2000–2008. Adult birds were captured upon their arrival, weighed and their tarsus length was measured. Males in their 3rd year or older were photographed for colour analysis of the breast ornament, which is a secondary sexual trait in this species: hue, saturation and brightness (HSB colour space) were measured (for a detailed description of ornament analysis see Albrecht et al. 2009). In all adults and 7 day old chicks a blood sample (20–30 μl) was collected and stored in 96% ethanol at −20° C until DNA extraction.

Genetic analyses

Genomic DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. A previous study on the structure and diversity of MHC in the Scarlet rosefinch revealed that there are 82 MHC class I variants (hereafter called 'alleles' for simplicity) in this population, with individuals displaying between three to nine alleles (mean: males = 5.15 ± 0.12 (SE), females = 5.1 ± 0.12 (SE)). Most of the adult birds displayed unique MHC class I genotypes (Promerova et al. 2009). We used single-strand conformation polymorphism (SSCP) analysis in a capillary to assess allelic diversity of the exon 3 region in MHC class I genes, which encodes parts of the peptide-binding region of the protein (for more details see Promerova et al. 2009). Alleles were visualized using GeneMapper v3.7 (Applied Biosystems). To assess paternity, we genotyped all individuals at 15 polymorphic microsatellite loci (amplification conditions used in this study are described in Poláková et al. (2007), for more details on parentage analysis see Albrecht et al. 2009). The genotypes were analysed using GeneMapper v3.7 (Applied Biosystems). The same panel of microsatellites and two additional loci were used for calculating Internal relatedness (IR; Amos et al. 2001) and standardized

heterozygosity (Het_{ST}; Coltman et al. 1999) using 'IR macroN3' (<www.zoo.cam.ac.uk/zoostaff/amos>).

Statistical analyses

Pairs were considered social if the mates took care of the young together (feeding, nest defence). Extra-pair (EP) were considered males with which females had young outside the pair-bond, and these males apparently did not contribute to parental care. Generalized linear mixed models (GLMM; R 2.8.1 <http://www.r-project.org/>) were used to assess the effect of allelic diversity (number of identified MHC class I alleles) of fathers on the occurrence of extra-pair paternity (EPP), since several males were sampled repeatedly over years. To find out whether females can gain indirect benefits from EP matings via increasing offspring MHC variability, the number of alleles per chick in within-pair (WPO) and extra-pair offspring (EPO) was compared, using nest identity as a random effect in the analysis (GLMM; R 2.8.1). We also tested if females might increase the number of different alleles in their broods via EPC, by comparing the overall number of different MHC class I alleles for the whole broods with and without EPP. To account for pseudoreplication arising from repeated inclusion of particular females in successive breeding seasons, female identity was included as a random effect in the analysis (GLMM; R 2.8.1).

MHC-similarity between males and females was calculated as MHC allele-sharing: the proportion of allele-sharing in a pair is twice the sum of alleles the individuals share divided by the sum of alleles of both individuals – $(D = 2F_{ab}/(F_a + F_b)$; Wetton et al. 1987). We tested whether females were more dissimilar in MHC from EP-males than from their social mates by comparing MHC allele-sharing between social and extra-pair mates, respectively (t-test, STATISTICA 6.0).

We tested for correlation between individual standardized heterozygosity on microsatellites (using both IR and Het_{ST}) and number of MHC alleles using Spearman's correlation test (STATISTICA 6.0).

Results

Effect of MHC variation on extra-pair mating

EPO were found in 37.8% of nests. We found significant negative effect of the number of MHC alleles in the social male on the occurrence of EPP in his own nest ($N = 104$ nests, GLMM, $\chi^2 = 7.3$, $DF = 1$, $p \leq 0.01$; slope: -0.573 ± 0.216 (SE); Fig. 1) independent of female MHC variability (GLMM, female MHC: $\chi^2 = 0.47$, $DF = 1$, $p = 0.49$; slope: 0.164 ± 0.230 (SE), interaction between M- and F-MHC: $\chi^2 = 1.19$, $DF = 1$, $p = 0.28$; slope: -0.210 ± 0.200 (SE); random factor = male identity). However, the cuckolded social male was not less MHC heterozygous than the male that had cuckolded on him (paired *t*-test: $p = 0.29$, $n = 36$ male couples). We compared MHC allele-sharing between females and their social and EP mates, respectively. There was no evidence supporting the idea of higher MHC dissimilarity of EP than social males ($p = 0.6$, $n = 34$ pairs of mating events). There was no correlation between MHC similarity of social pairs and occurrence of EPP (Spearman; $p = 0.8$, $n = 104$ mating events).

Finally we compared the number of MHC class I alleles of EPO and WPO for each nest with mixed paternity, and we found no difference in allelic diversity between the nestlings (GLMM, $p = 0.8$, $\chi^2 = 0.064$, $DF = 1$, slope: -0.021 ± 0.082; random factor = brood identity). To address the possibility that females increase the total number of MHC class I allels in their broods via EPP, we compared the number of alleles in nests containing EPO with nests without EPO in an analysis assuming Poisson distribution of the dependent variable (total number of different alleles). However, in a model containing brood size, total number of alleles in social partners and nest type (containing or not containing EPO) as explanatory variables, the latter was a poor predictor of the number of different alleles in broods ($n = 102$ nests, GLMM, effect of EPO occurrence in nest: $\chi^2 = 2.329$, $p = 0.127$, slope: 0.118 ± 0.078; effect of brood size: $\chi^2 = 2.736$, $p = 0.098$, slope: 0.056 ± 0.034; effect of the number of parental alleles: $\chi^2 = 14.234$, $p < 0.001$, slope: 0.099 ± 0.026).

Association of MHC diversity and phenotypic traits

We analysed whether some of the measured phenotypic traits could be affected by the number of MHC class I alleles to reflect male genotype. However, we failed to show any relation between individual MHC diversity and body weight (Spearman; F: $p = 0.4$, $n = 66$; M: $p = 0.16$, $n = 91$) or tarsus length (Spearman; F: $p = 0.7$, $n = 65$; M: $p = 0.09$, $n = 88$), neither in males nor in females. Moreover, there was no association between the number of MHC alleles and the level of expression of the carotenoid-based feather ornamentation in males (Spearman; hue: $p = 0.3$, saturation: $p = 0.6$, brightness: $p = 0.12$; $n = 91$).

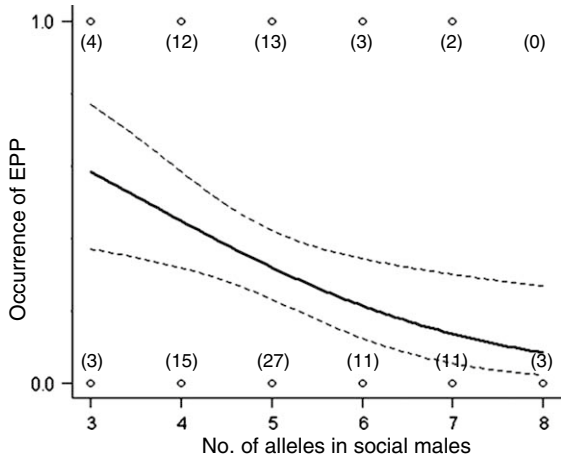

Figure 1. Predicted effect of MHC class I allelic diversity of social males on the occurrence of extra-pair paternity (EPP) in their nests. Counts of observations per each category of MHC class I allele numbers are given in parentheses. Dashed lines represent 95% CI. The lines are based on GLM, with occurrence of EPP in nests treated as binary variable (0 – no EPP detected, 1 – at lest one extra-pair offspring detected).

Comparing overall heterozygosity and MHC diversity

To exclude the probability that the individual MHC variability could be only a reflection of the overall heterozygosity, we tested if the number of MHC class I alleles in an individual is correlated with the individual's IR or Het_{ST}. We found no such pattern in the scarlet rosefinches (Spearman; IR: $p = 0.83$, $n = 614$; Het_{ST}: $p = 0.86$, $n = 614$).

Discussion

We tested for female extra-pair mate choice mechanisms in a socially monogamous songbird with biparental care. We found evidence that more MHC heterozygous males lose paternity in their own nest less frequently than males with low MHC variability. However, EP males were not more MHC-heterozygous than cuckolded social males, and EPO were not more MHC-heterozygous than WPO. Neither did the broods with EPO contain a higher number of different alleles than broods of entirely within-pair chicks. There was no support found for the complementarity hypothesis, but this might be partly due to the extreme diversity of MHC class I in this species and the impossibility of obtaining nucleotide sequences for all alleles. We think there is quite a high probability that some alleles are more similar in sequence to each other than others, thus testing of the complementarity hypothesis would be more robust with the data on sequences.

Hence, at least based on MHC class I variability there is no evidence for females gaining any indirect advantage from mating outside the pair-bond in the scarlet rosefinch. We also tested if these results might not only reflect a preference for overall heterozygosity, but the individual MHC-variability is not correlated to genome-wide heterozygosity in our dataset.

Our findings imply that the more MHC class I alleles a male has, the higher is the probability of protecting paternity in his own nest. Males which obtained extra-pair copulations were not more MHC-heterozygous than the males they cuckolded on, thus we suppose that females are unable to directly discriminate for more MHC-heterozygous males; they only seem to remain faithful to males with high MHC diversity. This might be due to better mate-guarding in the more MHC-heterozygous males (Zelano and Edwards 2002), but we cannot exclude the posibility that sperm-sperm and sperm-ova interactions contribute to the observed pattern. In any case, our study provides one of the first evidences for the effect of number of MHC class I alleles on within-pair fertilization success of males in birds.

In songbirds, there are so far only few studies reporting the impact of MHC genes on mating (Freeman-Gallant et al. 2003, Westerdahl 2004, Richardson et al. 2005, Bonneaud et al. 2006) and the results are largely equivocal. Moreover, only two of them focus on extra-pair mating. In their study in Seychelles warblers *Acrocephalus sechellensis*. Richardson et al. (2005) showed that EPP occurred when the social male was of low MHC diversity. However, unlike in the Scarlet rosefinches, in the Seychelles warblers the MHC diversity of the EP male was significantly higher than that of the cuckolded social male indicating a female preference for high MHC-heterozygosity. In Savannah sparrows *Passerculus sandwichensis*, Freeman-Gallant et al. (2003) found that yearling females (but not older) were more likely to obtain EPP if mated to a male with similar MHC to their own. Similar studies were conducted also in mammals. For example in the socially monogamous fat-tailed dwarf lemur *Cheirogaleus medius* females engaging in extra-pair copulations shared more MHC supertypes (allelic lineages grouped by functionality) with their social males than faithful females (Schwensow et al. 2008). In this study, nevertheless there was also evidence for 'good-genes-as-heterozygosity' hypothesis predicting mate choice in general for both social and extra-pair males, as the genetic fathers of offspring had more MHC supertypes than randomly chosen males (Schwensow et al. 2008).

The polygamous mating system precludes existence of any extra-pair copulations per se. Despite different scheme of pair forming and successive parental care the evidence obtained in polygamous fish may help to investigate female strategy concerning MHC variability in multiple mating. In the three-spined sticklebacks *Gasterosteus aculeatus* Eizaguirre et al. (2009) found that females preferentially mated with males with whom they shared an intermediate level of MHC diversity to produce offspring with optimal MHC heterozygosity (see also Milinski et al. 2005). At the same time males with certain MHC haplotypes ensuring resistance against common parasites were preferred (Eizaguirre et al. 2009).

Females may assess the genetic quality of males according to different cues. In mammals or fish, for instance, MHC has been shown to affect odour (Singh et al 1987, reviewed in Penn and Potts 1998), and through odour also sexual selection (in humans, Thornhill et al. 2003; mice, Penn and Potts 1998; fish, Milinski et al. 2010; lizards, Olsson et al. 2003). However, birds are presumed to rely more on visual cues and hearing than on olfaction (Roper 1999, but see also Balthazart and Taziaux 2009), although their olfactory receptors seem to be similar as in other vertebrates (Steiger et al. 2008). Alhough the relationship between condition-associated phenotypic traits and certain MHC genotype has been found in birds (von Schantz et al. 1997, Ekblom et al. 2004, Hale et al. 2009), in the Scarlet rosefinch we failed to find any correlation between the number of MHC class I alleles and condition-dependent traits such as body mass and tarsus length or expression of a secondary sexual ornamentation in males. This is despite the fact that the carotenoid-based feather ornament has already been proved to govern reproductive success in this species (Albrecht et al. 2009).

The molecular methods we used in this study are routinely used for analysing MHC in non-model species (e.g. Binz et al. 2001, Bryja et al. 2005, Alcaide et al. 2010, Baratti et al. 2010). When using the method of CE-SSCP, although the outcome is reliable genotyping, the particular nucleotide sequences remain unknown. Nevertheless, considering the impact of MHC on mate choice and phenotypic traits, it is possible that not only the number of alleles and identity of alleles is important, but also the actual nucleotide sequences and hence the structural differences among alleles. To conclude, in the future, new methods like 'next generation sequencing', which produce huge sets of sequence data (Babik et al. 2009) might

elaborate our understanding of the role of MHC in mate choice.

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