Cryptic preference for MHC-dissimilar females in male red junglefowl, Gallus gallus

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An increasing number of studies test the idea that females increase offspring fitness by biasing fertilization in favour of genetically compatible partners; however, few have investigated or controlled for corresponding preferences in males. Here, we experimentally test whether male red junglefowl, Gallus gallus, prefer genetically compatible females, measured by similarity at the major histocompatibility complex (MHC), a key gene complex in vertebrate immune function. Theory predicts that because some degree of MHC heterozygosity favours viability, individuals should prefer partners that carry MHC alleles different from their own. While male fowl showed no preference when simultaneously presented with an MHC-similar and an MHC-dissimilar female, they showed a ‘cryptic’ preference, by allocating more sperm to the most MHC-dissimilar of two sequentially presented females. These results provide the first experimental evidence that males might respond to the MHC similarity of a female through differential ejaculate expenditure. By revealing that cryptic male behaviours may bias fertilization success in favour of genetically compatible partners, this study demonstrates the need to experimentally disentangle male and female effects when studying preferences for genetically compatible partners.

Keywords: major histocompatibility complex; sexual selection; mate choice; genetic compatibility; sperm allocation; kin discrimination

1. INTRODUCTION

An enduring challenge in evolutionary biology is to elucidate the mechanisms that maintain polymorphism at fitness-related loci in the face of Darwinian selection. One such potential mechanism that has attracted increasing interest is mate preference based on the genetic complementarity of maternal and paternal genotypes, which is expected to evolve as a response to a number of selective advantages associated with, or conveyed by, offspring heterozygosity (Zeh & Zeh 1997; Trivers 1972; Burley 1977; Parker 1983; Jones & Hunter 1993; Owens & Thompson 1994; Johnstone et al. 1996; Amundsen & Forsgren 2001; Bonduriansky 2001; Kokko & Monaghan 2001; Johnstone et al. 2002; Chenoweth et al. 2006, 2007) and ‘cryptically’ through preferential ejaculate expenditure (Bonduriansky 2001; Reinhold et al. 2002; Wedell et al. 2002; Pizzari et al. 2003; Cornwallis & Birkhead 2006; Rubolini et al. 2006), particularly in species where sperm competition favours strategic sperm allocation (Wedell et al. 2002). It is well established that the relative number of sperm inseminated by competing males into a female is a reliable predictor of fertilization success in several species (e.g. Gage & Morrow 2003), including the fowl (Martin et al. 1974). Cryptic male preferences through differential male sperm investment in certain females may therefore influence fertilization success and represent a neglected source of variation in paternity.

Little is known about male mating preferences mediated by genetic compatibility. However, we do know that because of anisogamy, male preference functions may diverge from female preference functions: under some circumstances, males are selected to invest in genetically incompatible females while females are selected to resist fertilizations by genetically incompatible males, leading to sex-specific counteracting responses (Parker 1979; Ball & Parker 2003; Pizzari et al. 2004;
Kokko & Ots 2006; Parker 2006). In addition, we would expect male mating bias to be modulated by female availability, being more pronounced when different females are simultaneously, rather than sequentially, available to a male (Kokko & Ots 2006). Taken together, these factors indicate that the patterns of male preference can be both subtle and complex.

Studying male preferences for genetically compatible females is key to our understanding of selection on—and polymorphism of—fitness-related loci for two reasons. First, male preferences can potentially confound the study of biases in mating and fertilization success attributed to female preferences. For example, a bias in favour of genetically compatible fertilizations can be due to female preference, male preference or a combination of both. Similarly, lack of a fertilization bias may reflect lack of mating preference in both sexes or, alternatively, the outcome of counteracting strategies. Second, intersexual selection driven by male mating preferences is a neglected episode of sexual selection, and elucidating the evolution of male preferences is an important challenge in sexual selection theory (Wright et al. 2007; Pizzari & Bonduriansky 2009).

Here, we study overt and cryptic male preference for genetically compatible partners focusing on one of the best characterized candidates of genetic compatibility, the vertebrate major histocompatibility complex (MHC). MHC genes code for antigen-presenting molecules that are important in the acquired immune system (Janeway et al. 1999; Glick 2000). To some extent, more MHC diverse individuals may be more likely to resist a larger variety of parasites and pathogens than less MHC diverse individuals, either because of heterozygote advantage or because of increased likelihood of carrying MHC alleles that have a greater affinity for a particular pathogen (Penn & Potts 1999; Milinski 2006). A preference for MHC-dissimilar partners will maximize the MHC diversity of offspring and, therefore, improve their ability to cope with pathogens. Furthermore, because balancing selection maintains extreme MHC polymorphism, individuals sharing MHC alleles are likely to be genetically related (Brown 1983; Potts & Wakeland 1993; Brown & Eklund 1994; Penn & Potts 1999). Therefore, independent of pathogen resistance, preference for MHC-dissimilar partners may also mediate inbreeding avoidance (Brown 1983; Potts & Wakeland 1993; Brown & Eklund 1994; Penn & Potts 1999). Either way, by preferentially investing in MHC-dissimilar partners, individuals may increase offspring fitness.

While several studies have explored female preference for MHC-dissimilar males (e.g. Wedekind & Furi 1997; Olsson et al. 2003; Richardson et al. 2005; Schwensow et al. 2008), information on MHC-mediated mate choice in males is scarce and mostly limited to inbred lines of mice, in which evidence for male preference for MHC-dissimilar partners has been found by some (Yamazaki et al. 1976, 1978, 1988; Beauchamp et al. 1988), but not other studies (Eklund et al. 1991). In addition, there is some evidence that men preferred the odour of MHC-dissimilar women (Wedekind & Furi 1997). Male differential ejaculate expenditure according to the MHC similarity of a male with a female has not—to our knowledge—been experimentally tested. The objective of this study was to investigate the patterns of male mate choice and differential ejaculate expenditure in relation to MHC similarity during simultaneous and sequential exposure to females, in the sexually promiscuous red junglefowl, Gallus gallus.

We designed two experiments to test for differential mating response by a male based on his MHC similarity with a female, under high and low mate availability. The first experiment is a simultaneous mate choice experiment whereby the male is simultaneously presented to two females, an MHC-similar and an MHC-dissimilar female (high mate availability). The second experiment is a sequential mate choice experiment designed to test male mating response according to MHC similarity when females are sequentially presented to a male (low availability). We tested the following two predictions:

(i) males are more likely to mate with the MHC-dissimilar female and
(ii) males allocate more sperm to the MHC-dissimilar female.

2. MATERIAL AND METHODS
(a) Study species
The mating system of natural populations of red junglefowl including feral populations of its domestic subspecies, Gallus gallus domesticus, is characterized by the high opportunity for sexual selection that occurs both before and after copulation (Pizzari et al. 2002). Importantly, male fowl bias mating and ejaculate expenditure according to the reproductive quality of a female. First, males preferentially mate with and invest sperm in sexually novel females that they have not inseminated recently (Pizzari et al. 2003). Second, females display a sexual ornament, the comb, whose expression covaries, phenotypically and genetically, with different measures of female reproductive investment (Pizzari et al. 2003; Cornwallis & Birkhead 2007; Wright et al. 2007), and males preferentially mate with—and invest sperm in—females with larger combs (Pizzari et al. 2003; Cornwallis & Birkhead 2006). Finally, males show a tendency to avoid genetically related females when unrelated females are simultaneously available (Løvlie & Pizzari, submitted), but invest sperm in these females when unrelated females are not available (Pizzari et al. 2004).

(b) Study populations and MHC typing
The study was carried out over 3 years: 2005–2007. In 2005 and 2006, the study was conducted on a population of red junglefowl, G. gallus, housed at the Swedish University of Agricultural Sciences in Skara (Sweden). This Swedish population was founded from a zoo population originating from Thailand (Schütz et al. 2001). The birds were pedigree hatched, using methods identical to those of Pizzari et al. (2004), and pedigree data from two generations were available. In 2006, a random-bred stock of the Swedish population was established at the John Krebs Field Station of the University of Oxford, and in 2007, the study was continued on the UK population. No pedigree data were available for this population.

All birds from both the Swedish and Oxford populations were typed for MHC class I and II alleles. The MHC structure of the fowl is well known and simple compared with mammals (Kaufman et al. 1999; Jacob et al. 2000; Wallin et al. 2006; Shaw et al. 2007). The MHC B haplotype
appears to be inherited as a single unit and contains two MHC class I loci (BF1 and BF2) and two MHC class II loci (BLB1 and BLB2), all encoding antigen-binding molecules (Kaufman et al. 1999). The loci BF2 and BLB2 have been shown to express far more than the BF1 and BLB1 loci, respectively, and there is evidence that these dominantly expressed loci determine the immune response to certain infectious pathogens (Kaufman et al. 1999; Wallny et al. 2006). Thus, BF1 and BLB1 have been termed the minor MHC class I and II loci, respectively, and BF2 and BLB2 have been termed the major MHC class I and II loci, respectively (Kaufman et al. 1999; Jacob et al. 2000; Wallny et al. 2006; Shaw et al. 2007).

Several alternative methods have been developed to screen individual genetic variation directly in the domestic chicken, including single-strand conformation polymorphism (Goto et al. 2002), automated sequencing (Livant & Ewald 2005) and by using adjacent microsatellites variation (Fulton et al. 2006). All these methods have different advantages, but we used the alternative method of reference strand conformation analysis (RSCA). As an automated method, RSCA is suitable for screening large populations reliably, with excellent between-run repeatability even for large fragments (less than 500 bp), and can separate alleles that differ by a single nucleotide (Ramon et al. 1998; Kennedy et al. 2002).

Four class I and four class II fluorescently labelled reference strands were used for RSCA genotyping, all originating from cloned domestic fowl alleles (further details including GenBank accession numbers can be found in Worley et al. 2008). Class I fragments were initially amplified using the primers C71 (5′-CGAGCTCTCACCCCTGTGGTGTTCTCTG-3′) and C75 (5′-CTCTGTGAGGCATAGCCTTC-3′) developed from domestic chicken lines (Shaw et al. 2007). Amplicons comprise 767 bp fragments encompassing exons 2 and 3 of both minor and major loci. A 277 bp exon 2 fragment of class II major and minor loci was amplified using the primers OL284BL (5′-GTGCGCGCAGCGTATC-3′) and RV280BL (5′-TCCTCCTCAGGGCTGAAAGG-3′) (Goto et al. 2002). All amplification conditions have been described in Worley et al. (2008).

A nested PCR protocol was then devised to allow us to resolve each separate locus. The primers C477 (5′-CTCCTGCCCAGCTCAGC-3′) and RV280BL (5′-CCATGCCTTGCAGAAAT-3′), which amplify a 3.4 kB fragment spanning exons 2 and 3 of both minor and major loci, were used to amplify a fragment of approximately 3.6 kbp containing only the class I minor locus (Shaw et al. 2007). A second PCR was then performed on the class I minor amplicons using the non specific BF primers C71 and C75. Similarly, the class II minor locus was amplified by the primers C275 (5′-GGTCTACCAAAAGGGTCTTCTCGTGCTACACTT-3′) and C243 (5′-CCATGCGCTGATCATG-3′), which amplify a 3.4 kbp fragment spanning the adjacent B-lec and Tapasin genes (Jacob et al. 2000). Again, a second PCR was performed on these class II minor amplicons using the non specific primers OL284BL and RV280BL as above. All amplification conditions have been described in Worley et al. (2008). Therefore variation at all four MHC B loci could be identified independently, which enables the reconstruction of the overall MHC haplotype without the assumption of non-recombination.

The Swedish population of 84 individuals contained 9 alleles across the two class I loci (six major and three minor) and 10 alleles across the two class II loci (five major and five minor). The four MHC B loci associated into distinct MHC haplotypes, with no evidence of recombination between loci. Sequence polymorphism was similar to that of domestic fowl at 0.068 for class I and 0.108 for class II loci ( Worley et al. 2008; table 3) and population expected heterozygosity was variable across loci (class I: minor = 0.57, major = 0.72; class II: minor = 0.65, major = 0.68). The degree of MHC similarity between two individuals was calculated using the following formula: degree of MHC similarity is equal to (2x)/n, where x is the number of alleles shared between the two individuals and n is the total number of alleles present in the two individuals.

(c) General experimental protocol

Each male was kept physically isolated from females for at least 48 h prior to each experimental trial in order to control for sperm depletion and test all males with maximal extragonadal sperm reserves (Etches 1996). In each trial, a male was exposed to females held by trained staff. To control for holder effects on male responses to females, females were presented to the male from behind a partition that enabled the male free access to the females but prevented visual contact with the holder. A male was given 1 min to familiarize himself with the females, before females were placed in a soliciting position to enable mating. Manual holding of the female in a soliciting position triggers male mounting and controls for differential female responses to the male, which may influence male sexual behaviour (e.g. Pilastro et al. 2004). During each trial, females were fitted with a harness over the cloaca, which enabled the collection of each ejaculate (Pizzari et al. 2003, 2004). The ejaculate volume allocated to the female was collected and recorded to the nearest 5 μl with a pipette. The number of sperm contained in an ejaculate was calculated using standard techniques (Bakst & Cecil 1997; Pizzari et al. 2003). Each female was weighed to the nearest 0.1 g and female comb height and length measured to the nearest 0.01 mm with a digital calliper. Female comb area was measured as (x × y)/2, where x is female comb height and y is comb length. We minimized phenotypic differences in comb size and body mass between the two females exposed to the same male, and also controlled for their potential effects statistically (see below).

All analyses were performed using generalized linear mixed models (GLMMs) with restricted maximum-likelihood (REML) estimation in SAS v. 9.1 (Wolfgang & O’Connell 1993). We selected the minimum adequate model using a sequential addition of variables, whereby the model initially contains no variables and variables are added sequentially (Grafen & Hails 2002). At each step, the variable with the lowest p and highest F values was inserted into the model and only variables with p < 0.05 were added into the model (Grafen & Hails 2002). The significance of fixed effects was examined using Wald-type tests (type III for main effects and type I for interactions; Grafen & Hails 2002).

(d) Simultaneous mate choice and sperm allocation

This experiment was carried out on 27 males, 11 in 2006 and 16 in 2007. In total, 28 females were used, 12 in 2006 and 16 in 2007. A male and female were considered MHC-dissimilar if the total number of MHC alleles shared was less than 0.18 and similar if more than 0.57. In 2006, each male did not
share grandparents with either female within each trial \((r<0.0625)\). In 2007, pedigree data were not available and relatedness between individuals was unknown. When pairing females, differences in comb size and mass were minimized. Female comb length, comb height and mass did not significantly differ between MHC-similar and MHC-dissimilar females within each experimental pair (paired \(t\)-test; female comb length: \(n\) pairings \(=27\), \(T = -0.16\), \(p = 0.874\); female comb height: \(n\) pairings \(= 27\), \(T = 0.79\), \(p = 0.440\); female mass: \(n\) pairings \(= 27\), \(T = -0.34\), \(p = 0.736\)). In addition, to control for differences in female comb size and body mass between the two females of a trial, we standardized the value of each of the two females used within a trial against the average value for that trial (e.g. comb size of female \(x\) was calculated as: \((x-y)/(x+y)/2\), where \(x\) is the comb size of female \(x\) and \(y\) is the comb size of female \(y\).

The two females were first simultaneously exposed to a male for 1 min in an upright position to allow him to familiarize himself with the females. Females were then presented simultaneously to the male in a soliciting position for 20 min or until he copulated with one of the females. In two instances, the male did not copulate with either female within 20 min, and the trial was repeated, at least 48 h later. The female with whom the male copulated first was considered the ‘preferred’ female of the two. A male was deemed to have copulated with a female if cloaca contact was observed, and sperm allocation was measured by the number of sperm ejaculated by the male. If a male copulated with a female but did not ejaculate any sperm, sperm allocation to this particular copulation was recorded as zero. Immediately after the first presentation, the male was presented once again with the same two females but this time a wired cage was placed over the preferred female, that prevented physical but not visual contact with her, and forced the male to copulate with the female with whom he did not copulate in the first presentation (‘least preferred’ female, see: Pizzari et al. 2003).

To control for copulation order effects on sperm allocation, a second trial was conducted with the same females after a male was sexually rested. In the second trial, males were forced to copulate in the reverse order of the first trial: with the least preferred female first and the preferred female second. Therefore, in the second trial, a male was again given the possibility to copulate with each female, but in this case copulation order was predetermined and reversed compared to his first trial. Once again, the amount of sperm allocated to each female was quantified.

We carried out the analysis in two parts. First, we tested whether males preferentially copulate with an MHC-dissimilar female (i.e. whether they copulated first with the MHC-dissimilar female in the first trial) through a \(\chi^2\)-test. Second, we investigated whether the number of sperm in the ejaculate allocated to each female varied according to MHC similarity through a GLMM with residual pseudo-likelihood estimation, a Poisson error distribution and log link function, forward selection of non-significant terms, the number of sperm allocated to a female in a trial as the dependent variable, MHC similarity category (similar or dissimilar) and copulation order (first or second with female) as fixed factors. Standardized female comb size and standardized female mass were entered as covariates, while trial (first allocation or second allocation trial), male identity (nested within trial), female identity and the interaction of male and female identity and year of experiment were entered as random factors. The fixed effect with the highest \(p\) value was sequentially added until only significant terms \((p>0.05)\) remained in the model.

In order to explore more fully the possibility that any MHC similarity effect could be mediated by a single area or a subset of the MHC, and determine the extent to which the two MHC classes contribute to such effects, we replaced the index of MHC similarity in the model with indices of similarity based on the subsets of the MHC loci, i.e. (i) the number of alleles shared by a male and a female in MHC class I and (ii) class II separately, (iii) the combined number of MHC major alleles shared, (iv) the number of MHC major alleles shared in class I and (v) class II separately. Finally, MHC genes are thought to be inherited as a unit (i.e. a haplotype) as recombination between the class I and II genes was not observed in over 6000 domestic fowl progeny (Skjødt et al. 1985). We also observed no evidence of recombination in the jungle fowl populations, as independently amplified MHC loci associated into distinct haplotypes. Consequently, genes tightly linked to the MHC genes, such as olfactory receptor (OR) genes, may mediate MHC haplotype recognition rather than individual MHC allele recognition (Penn & Potts 1999). We, therefore, also tested the effect of MHC similarity measured as the proportion of total number of haplotypes shared. Because two birds can only share both, one or zero haplotypes, this index had only three values: 1.0, 0.5 and 0.0, respectively. It is important to note that during forward selection of explanatory variables in the models, different MHC similarity measures were considered as alternative explanatory variables owing to their collinearity. Therefore, different MHC similarity measures were never entered simultaneously in the same model. Akaike information criterion (AIC, Akaike 1973, 1987; Bozdogan 1987, 2000; SAS 2006) values were used to compare the adequacy of alternative models in which different MHC similarity measures were significant explanatory variables. The difference in AIC values between these models was considered significant when greater than 1 (Akaike 1973, 1987; Bozdogan 1987, 2000; SAS 2006).

(e) Sequential mate choice and sperm allocation
This experiment was conducted on 27 males, 14 in 2005, 10 in 2006 and 8 in 2007 (some males were used both in 2005 and 2006). In total 42 females were used, 21 in 2005, 17 in 2006 and 10 in 2008 (six females were used both in 2005 and 2006). In each trial, a male was exposed to a single female and allowed to copulate with her. We measured male propensity to copulate as the time (to the nearest 0.5 min) it took a male to copulate with a female immediately after she was presented to him. Males sometimes mount females without allocating any sperm to the female (Pizzari et al. 2003; Lovlie et al. 2005). To control for this possibility, males were allowed to copulate up to two times with a female during a trial of 20 min.

First, we investigated male propensity to copulate in relation to MHC similarity, by analysing the time elapsed to first copulation in relation to MHC similarity through a REML GLMM with a lognormal error distribution and identity link function, time elapsed to first copulation as the dependent variable, the MHC similarity (see below), female mass and female comb area as covariates and male and female identity and year of experiment as random factors. Second, we investigated male differential ejaculate expenditure according to MHC similarity, by analysing the number of
Table 1. Sources of variance in the number of sperm allocated by a male to a female in the simultaneous mate choice experiment ($n_{males}=27$), degrees of freedom (d.f.), $F$ ratio ($F$) and significance level ($p$). (Values in bold represent $p$-values < 0.05.)

<table>
<thead>
<tr>
<th>model</th>
<th>d.f.</th>
<th>$F$</th>
<th>$p$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MHC category</td>
<td>1, 26</td>
<td>3.01</td>
<td>0.094</td>
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<tr>
<td>copulation order</td>
<td>1, 26</td>
<td>34.69</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>standardized female comb</td>
<td>1, 26</td>
<td>0.66</td>
<td>0.422</td>
</tr>
<tr>
<td>size</td>
<td>1, 26</td>
<td>0.21</td>
<td>0.653</td>
</tr>
<tr>
<td>standardized female mass</td>
<td>1, 52</td>
<td>2.37</td>
<td>0.13</td>
</tr>
</tbody>
</table>

sperm in the ejaculate allocated to each female according to MHC similarity through a REML GLMM with a Gaussian error distribution and identity link function, the number of sperm allocated to a female in a trial as the dependent variable, the number of copulations during trial (one or two copulations) as fixed factors; male and female identity and year of experiment as random factors; female mass, female comb area and MHC similarity, measured as the number of MHC alleles shared by a male and a female across both major and minor loci of each class combined, as covariates. Once again, we further explored the mechanisms underlying the potential effects of MHC similarity by replacing MHC similarity in the model with (i) the number of alleles shared by a male and a female in MHC class I and (ii) class II separately, (iii) the combined number of MHC major alleles shared, (iv) the number of MHC major alleles shared in class I and (v) class II separately and (vi) the proportion of total number of haplotypes shared, as outlined above.

We carried out each analysis (i.e. mating propensity and ejaculate expenditure) using the data collected from both the Swedish population and the UK population. We then repeated each analysis focusing exclusively on the data collected from the Swedish population, for which pedigree data were available, entering pedigree relatedness (a male and a female were either full-sib, $r=0.5$, or unrelated, $r<0.0625$, to each other) as an additional fixed factor, which enabled us to control for the potential effects of relatedness.

3. RESULTS

(a) Simultaneous mate choice and sperm allocation

Males were not more likely to initiate a copulation with an MHC-dissimilar female first ($X^2=0.054; p=0.816$); 13 males copulated with the MHC-similar female first and 14 males copulated with the MHC-dissimilar females first.

After controlling for copulation order, males did not allocate sperm differentially according to their MHC similarity with a female (table 1).

(b) Sequential mate choice and sperm allocation

Males did not show a higher propensity to copulate with MHC-dissimilar females (table 2a). This result was confirmed when considering only the data from the Swedish population.

After controlling for genetic relatedness between a male and a female, female comb size and body mass, we did not find a significant relationship between male propensity to copulate and MHC similarity (table 2b).

Males allocated significantly more sperm to the female with whom they were most MHC-dissimilar (table 3a; figure 1). All measures of MHC similarity were significant when the effect of each was entered separately into the model. However, based on the comparison of AIC values between the models, the measure of MHC similarity that explained the most variance was the total number of MHC haplotypes shared by a male and a female ($F_{1,11}=6.97; p=0.023$; figure 1). This result was not significant when analysing the smaller dataset from the Swedish population only (table 3b).

4. DISCUSSION

This study investigated patterns of overt and cryptic male mating preferences in relation to the MHC similarity of a female, during both sequential and simultaneous mate choice. We found no evidence of overt male mating preference. However, while the males were just as likely to mate with an MHC-dissimilar as with an MHC-similar female, they showed some cryptic preference by allocating more sperm to MHC-dissimilar females during sequential mate choice.

Previous work has demonstrated male mating preferences in the fowl. First, male feral fowl, G. g. domesticus, are more likely to mate with a female with a larger comb (Cornwallis & Birkhead 2007), a trait phenotypically (Pizzari et al. 2003; Cornwallis & Birkhead 2007) and genetically (Wright et al. 2007) correlated with female reproductive investment. Second, male red junglefowl display kin recognition and avoid full-sib sisters when unrelated females are available (Lavlie & Pizzari submitted), but eagerly mate with full-sib sisters when unrelated females are not available (Pizzari et al. 2004; Lavlie & Pizzari submitted). However, the full extent of male preference based on female comb size and genetic relatedness is revealed only when considering ‘cryptic’ male preferences in terms of differential ejaculate expenditure (Pizzari et al. 2003, 2004; Cornwallis & Birkhead 2007). Consistent with previous work, the results of the present study suggest that male fowl might show a preference for MHC-dissimilar partners but only in terms of ejaculate expenditure and only when a male encounters individual females sequentially.

While the proximate mechanisms underlying recognition of MHC similarity are unknown in birds, studies in other vertebrates suggest that it may be mediated by olfactory cues (e.g. Fan et al. 1995; Wedekind et al. 1995; Wedekind & Furi 1997; Yamazaki et al. 1999; Reusch et al. 2001; Carroll et al. 2002; Aeschlimann et al. 2003; Olsson et al. 2003; Hurst et al. 2005; Milinski et al. 2005). For example, both mice (Yamazaki et al. 1999; Carroll et al. 2002) and rats (Brown et al. 1987) are able to discriminate between the urinary odour of an MHC-dissimilar and an MHC-similar individual, although in mice there seems to be a learnt component to MHC recognition (Beauchamp & Yamazaki 2003; Hurst et al. 2005). As in mice, MHC recognition in humans appears to be mediated by odour cues because both males and females prefer the odour of MHC-dissimilar individuals (Wedekind et al. 1995; Wedekind & Furi 1997) and polymorphic OR genes are located in the MHC (Fan et al. 1995). Another example of odour-mediated MHC recognition was provided by a study of sand lizards, Lacerta agilis, which experimentally demonstrated that the females preferred to associate with odour samples...
Table 2. Sources of variation in mate preference in the sequential experiment for (a) the overall dataset ($n_{males} = 27$) and (b) the Swedish population only ($n_{males} = 19$). (AIC value was used to select the best model, with smaller AIC indicating a better-fit model. The difference between the two AIC values was considered insignificant when equal to or less than 1.)

<table>
<thead>
<tr>
<th>model</th>
<th>AIC</th>
<th>d.f.</th>
<th>$F$</th>
<th>p-value</th>
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<tr>
<td>(a) the overall dataset ($n_{males} = 27$)</td>
<td></td>
<td></td>
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<tr>
<td>MHC alleles shared$^a$</td>
<td>307.2</td>
<td>1, 12</td>
<td>0.29</td>
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<td>MHC haplotypes shared</td>
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<td>0.574</td>
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<td>female comb size</td>
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<td>1, 13</td>
<td>1.44</td>
<td>0.251</td>
</tr>
<tr>
<td>female mass</td>
<td>304.96</td>
<td>1, 13</td>
<td>0.48</td>
<td>0.502</td>
</tr>
<tr>
<td>(b) the Swedish population only ($n_{males} = 19$)</td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>relatedness</td>
<td>215.75</td>
<td>1, 4</td>
<td>5.81</td>
<td>0.074</td>
</tr>
<tr>
<td>MHC alleles shared$^b$</td>
<td>214.27</td>
<td>1, 3</td>
<td>0.04</td>
<td>0.847</td>
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<td>MHC haplotypes shared</td>
<td>206.28</td>
<td>1, 3</td>
<td>0.03</td>
<td>0.875</td>
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<tr>
<td>female comb size</td>
<td>224.6</td>
<td>1, 4</td>
<td>2.27</td>
<td>0.206</td>
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<tr>
<td>female mass</td>
<td>211.57</td>
<td>1, 3</td>
<td>1.04</td>
<td>0.383</td>
</tr>
</tbody>
</table>

$^a$Alternative MHC similarity measures, MHC class I alleles shared, MHC class II alleles shared, MHC major alleles shared, MHC major I alleles shared and MHC major II alleles shared did not significantly predict mate preference in the sequential experimental.

Table 3. Sources of variation in the number of sperm ejaculated by a male in the sequential experiment. (a) The overall dataset ($n_{males} = 27$) and (b) Swedish population only ($n_{males} = 19$). AIC values were used to select the best model, with smaller AIC indicating a better-fit model. The difference between the two AIC values was considered insignificant when equal to or less than 1. Values in bold represent p-values < 0.05.

<table>
<thead>
<tr>
<th>model</th>
<th>AIC</th>
<th>d.f.</th>
<th>$F$</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) the overall dataset ($n_{males} = 27$)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>total MHC alleles shared$^a$</td>
<td>2983.92</td>
<td>1, 11</td>
<td>5.82</td>
<td>0.035</td>
</tr>
<tr>
<td>MHC haplotypes shared$^b$</td>
<td>2982.87</td>
<td>1, 11</td>
<td>6.97</td>
<td>0.023</td>
</tr>
<tr>
<td>comb size</td>
<td>2957.91</td>
<td>1, 11</td>
<td>0.77</td>
<td>0.401</td>
</tr>
<tr>
<td>female mass</td>
<td>2942.52</td>
<td>1, 11</td>
<td>2.77</td>
<td>0.124</td>
</tr>
<tr>
<td>(b) Swedish population only ($n_{males} = 19$)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>relatedness</td>
<td>2075</td>
<td>1, 4</td>
<td>1.19</td>
<td>0.336</td>
</tr>
<tr>
<td>total MHC alleles shared$^c$</td>
<td>2079.68</td>
<td>1, 4</td>
<td>1.24</td>
<td>0.328</td>
</tr>
<tr>
<td>MHC haplotypes shared$^c$</td>
<td>2078.68</td>
<td>1, 4</td>
<td>2.36</td>
<td>0.199</td>
</tr>
<tr>
<td>comb size</td>
<td>2093.01</td>
<td>1, 4</td>
<td>0.04</td>
<td>0.843</td>
</tr>
<tr>
<td>female mass</td>
<td>2077.82</td>
<td>1, 4</td>
<td>1.21</td>
<td>0.333</td>
</tr>
</tbody>
</table>

$^a$When comparing AIC values between the models no other alternative MHC similarity measures, MHC class I alleles shared, MHC class II alleles shared, MHC major alleles shared, MHC major I alleles shared and MHC major II alleles shared were a significantly better fit in explaining variation in number of sperm ejaculated when using the overall dataset.

$^b$MHC haplotypes shared explained significantly more of the variance in the number of sperm ejaculated than all other MHC similarity measures, MHC class I alleles shared, MHC class II alleles shared, MHC major alleles shared, MHC major I alleles shared and MHC major II alleles shared, when comparing AIC values and using the overall dataset.

$^c$Alternative MHC similarity measures, MHC class I alleles shared, MHC class II alleles shared, MHC major alleles shared, MHC major I alleles shared and MHC major II alleles shared did not significantly predict variation in the number of sperm when restricting the analysis to the Swedish population.

obtained from MHC-dissimilar males at the class I loci (Olsson et al. 2003). The fowl MHC exhibits a low level of recombination, giving rise to distinct haplotypes that are relatively conserved in evolution (Skjødt et al. 1985; Kaufman et al. 1999). Therefore, genes tightly linked to the MHC B region, such as olfactory genes, may mediate MHC genotype recognition (Penn & Potts 1999). It has recently been found that there are 218 non-identical fowl genes that are predicted to be orthologous to one of two OR gene clusters in humans, which are positioned next to the MHC class I gene clusters (International Chicken Genome Sequencing Consortium 2004). Furthermore, there are at least 554 OR genes (Aloni et al. 2006), challenging the traditional view that birds have a poor sense of smell (Alcock 1989). The high number of OR genes may indicate that olfactory cues are important in the fowl. The fact that the measure of MHC similarity, which explained most of the variance in terms of ejaculate expenditure, is the number of MHC haplotypes shared by a male and a female supports the hypothesis that MHC recognition may be mediated by alleles closely linked to the MHC B complex.

Theory predicts individuals to prefer reproductive partners of different MHC genotypes (Penn & Potts 1999; Milinski 2006). In addition to MHC heterozygosity, a preference for MHC-dissimilar partners may also be explained by inbreeding avoidance (Brown & Eklund 1994). We used the dataset of the Swedish population, for which MHC and genetic relatedness information was available, to attempt to disentangle the effects of MHC similarity per se from the effect of inbreeding avoidance. The effect of MHC similarity detected in the sequential mate choice experiment disappeared when we restricted the analysis to the Swedish population. However, it is debatable whether this result can be explained by the effects of genetic relatedness for two reasons. First, we did not detect a relatedness effect in this dataset. Second, previous work on this population indicated that males show only a weak
tendency to inseminate more (rather than fewer) sperm when exposed to full-sib sisters. Because related individuals are more likely to have similar MHC, this response would counteract rather than explain the male cryptic preference for MHC-dissimilar females detected by the analysis of the whole dataset. Instead, it is possible that restricting the analysis to a subset of the dataset may have reduced its power, preventing its ability to detect relatedness and MHC-similarity effects.

Theory would also predict males to become more selective as the availability of females increases (Parker 1983; Sullivan 1994; Deutsch & Reynolds 1995; Johnstone et al. 1996; Kokko & Monaghan 2001; Kokko & Otis 2006), and thus we would expect this preference to be more pronounced under simultaneous rather than sequential mate choice. Contrary to this expectation, we found that males biased ejaculate expenditure when they were exposed to only one (rather two) female at a time. A potentially confounding factor may be the social status of a male, which has been shown to influence strategies of differential ejaculate expenditure in this species (Pizzari et al. 2003; Cornwallis & Birkhead 2006). Dominant males are often exposed to lower risk and intensity of sperm competition, and will bias ejaculate expenditure in favour of the more ornamented of two simultaneously presented females, while this bias disappears when females are presented sequentially, as predicted by the theory (Cornwallis & Birkhead 2006). Subdominant males, on the other hand, typically experience limited access to females and do not bias ejaculate expenditure under simultaneous nor sequential female exposure (Cornwallis & Birkhead 2006). However, our study tested individual males singly, in isolation from competitors when any influence of status on ejaculate expenditure tends to disappear (e.g. Pizzari et al. 2003), and the present results are therefore unlikely to be influenced by social status. Clearly, the adaptive significance of the patterns of cryptic male preference revealed by this study warrants further research.

Regardless of the underlying functional mechanisms, the possibility that males bias ejaculate expenditure in relation to their genetic compatibility with a female has some important implications for the study of female preferences. Three lines of arguments have been interpreted as evidence that females prefer MHC-dissimilar males. First, mate choice trials have shown some evidence for higher female propensity to mate with MHC-dissimilar partners in a range of species (e.g. humans: Wedekind et al. 1995; Ober et al. 1997; Wedekind & Furi 1997; rodents: Arcaro & Eklund 1998; Jordan & Brufard 1998; Penn & Potts 1999; fishes: Landry et al. 2001; Reusch et al. 2001; Aeschlimann et al. 2003; Wegner et al. 2003; Milinski et al. 2005; Jager et al. 2007; sand lizards, L. agilis: Olsson et al. 2003). Second, in some natural populations, females are more likely to form social bonds with MHC-dissimilar partners, and/or seek extra-pair copulations with males that are more MHC-dissimilar than their social partners (e.g. passerine birds: Freeman-Gallant et al. 2002; Richardson et al. 2005; Bonneaud et al. 2006). Third, paternity is sometimes biased in favour of MHC-dissimilar parents (e.g. Atlantic salmon, Salmo salar: Consuegra & de Leaniz 2008; passerines: Freeman-Gallant et al. 2003; Richardson et al. 2005; grey mouse lemur, Microcebus murinus: Schwensow et al. 2008). However, the relative number of sperm inseminated by competing males into a female is a well-known predictor of fertilization success under sperm competition in the fowl (Martin et al. 1974), as in several other species (e.g. Gage & Morrow 2003). Biases in male ejaculate expenditure are, therefore, likely to influence fertilization success and variation in paternity might be influenced by cryptic male preference, thus calling into question the interpretation of the second and third approaches outlined above.

More generally, the patterns of variation in paternity within broods are often used to study cryptic female choice, particularly in relation to genetic compatibility. While the functional significance of female remating remains debated (Pizzari & Snook 2004; Arnqvist & Kirkpatrick 2005; Akcay & Roughgarden 2007; Griffith 2007), the results of our study indicate that males too can cryptically bias fertilization success in relation to the genetic compatibility of a female. Future studies would need to experimentally disentangle the effect of male- and female-driven bias in paternity to better understand mate preferences and their functional implications.

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