Female bluethroats enhance offspring immunocompetence through extra-pair copulations

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Female birds frequently copulate with extra-pair males1,2, but the adaptive value of this behaviour is poorly understood3. Some studies have suggested that ‘good genes’ may be involved, where females seek to have their eggs fertilized by high-quality males without receiving any material benefits from them4,5. Nevertheless, it remains to be shown that a genetic benefit is passed on to offspring6,7. Here we report that nesting bluethroats, Luscinia svecica, sired by extra-pair males had a higher T-cell-mediated immune response than their maternal half-siblings raised in the same nest. The difference could not be attributed to nesting body mass, sex or hatching order, but may be an effect of paternal genotype. Extra-pair young were also more immunocompetent than their paternal half-sibs raised in the genetic father’s own nest, which indicates an additional effect of maternal genotype. Our results are consistent with the idea that females engage in extra-pair copulations to obtain compatible viability genes, rather than ‘good genes’ per se.

Extra-pair mating systems are highly suitable for the study of genetic benefits of mate choice. When eggs in a clutch are fertilized by both the social male and one or more extra-pair males, the effect of paternal genes on offspring fitness can be assessed directly by comparison of maternal half-sibs6. This is because the sib groups share the same rearing environment and genes from the mother. The test prediction from ‘good genes’ models is then that extra-pair young (EPY) should perform better than within-pair young (WPY) raised in the same brood. Moreover, if females choose compatible male genes in extra-pair mate choice, it follows that EPY should also perform better than their paternal half-sibs, that is, the WPY of the extra-pair male. Here we have tested these predictions in a population of wild bluethroats where extra-pair paternity occurs at a relatively high frequency7,8.

We studied cell-mediated immunity in nesting bluethroats by a subcutaneous injection of phytohaemagglutinin (PHA) in one

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wing. PHA causes a local swelling response which peaks after 24 hours and which reflects T-cell reactivity. A genetic component of the PHA response has been documented in cross-foster studies of nestling passerines. It has also been shown that the nestling PHA responses correlate with subsequent survival and longevity.

There was a positive and nearly significant correlation between the PHA response and nestling body mass, using mean values for broods (r = 0.22, n = 75, P = 0.063). An influence of body mass on nestling PHA response has been documented in other passerines. Because we were interested in the genetic component of the PHA response, we used the residuals from a linear regression of wing swelling on body mass for individual nestlings (r = 0.18, n = 372, P = 0.0007) as our measure of immune response.

We found unequivocal support for the prediction that EPY are more immunocompetent than their maternal half-sibs raised in the same nest. EPY and WPY in broods of mixed paternity differed significantly in immune response (Table 1). The response was higher in EPY than in WPY in 24 of the 32 sets of maternal half-sibs (Fig. 1a). The mean difference (EPY – WPY) amounted to 0.51, expressed in phenotypic standard deviations of WPY. EPY and WPY did not differ in body mass growth or tarsus length growth during their first 8 days of the nestling period (Table 1). Hence, genes of extra-pair males appeared to enhance offspring immunocompetence without any corresponding effects on nestling body mass.

Although the underlying genetic mechanism for enhanced immunocompetence of EPY is still unknown, a genetic explanation can only be supported through the elimination of potentially confounding, non-genetic factors. For example, EPY might have been able to mount a stronger immune response if they were generally older, that is, they hatched earlier, than their WPY nest mates. This possibility can be rejected as EPY and WPY did not differ in order of hatching rank in broods where the PHA response was measured (F1,30 = 0.28, P = 0.60), nor in all mixed-paternity broods in our sample (F1,37 = 0.02, P = 0.89). Another potential bias might exist if immune responses differed between sexes and the more responsive sex were over-represented among EPY. This idea does not apply either as immune responses did not differ between male and female nestlings in mixed-paternity broods (F1,31 = 0.347, P = 0.56), nor in all mixed-sex broods (F1,62 = 0.138, P = 0.71). The sex ratio did not differ between EPY and WPY (exact permutation test on within-nest differences: n = 41, P = 0.43). Differential investment in EPY and WPY by parents also seems unlikely, as no evidence exists that parent birds, including bluethroats, can discriminate between their own and extra-pair offspring in a brood. There is a theoretical possibility that females can allocate more maternal resources into EPY eggs within a clutch, as female birds can alter the sex ratio or increase testosterone or material investments in clutches when socially paired to attractive mates. It remains to be shown, however, that females possess an ability to invest differentially in eggs of different sires within a clutch. If they do, then the only benefit that can select for this behaviour is a difference in offspring reproductive value caused by paternal genes.

We were able to assign paternity to 75% of all EPY in our data set, and 27 of the 84 males had EPY in other males’ nests. Identified cuckolders had a similar loss of paternity in their own nest (mean = 23% of brood) to that of males without any EPY (mean = 32% of brood, Mann-Whitney U-test: Z = –1.32, n1 = 27, n2 = 57, P = 0.19). This is apparently inconsistent with the traditional ‘good genes’ models, where females are assumed not to differ in mating preferences. Furthermore, we have not found any evidence for directional female preferences for male phenotypic traits, as revealed by analyses of male fertilization success in relation to various measures of body size and throat colouration (A.J. and J.T.L., manuscript in preparation). For 14 males, immune responses were measured in both their EPY and WPY. There was a significant effect of paternity on the immune response (Table 1). EPY were more immunocompetent than WPY in 12 of the 14 sets of paternal half-sibs (Fig. 1b). The mean difference (EPY – WPY) amounted to 1.44, expressed in phenotypic standard deviations of the WPY. In these comparisons, it should be noted that EPY and WPY were raised in different nests so that environmental differences might have influenced the results. We found, however, that the immune response correlated with subsequent survival and longevity.

**Table 1 Analyses of variance of immune response and growth in bluethroat nestlings**

<table>
<thead>
<tr>
<th>Test</th>
<th>Effect</th>
<th>Variance component</th>
<th>% of total variance</th>
<th>Mean square</th>
<th>d.f.</th>
<th>F</th>
<th>P</th>
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<tr>
<td>Maternal half-sib comparisons</td>
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<td>Nest</td>
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<td>38.5</td>
<td>0.5202</td>
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<td>Body mass growth</td>
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<td></td>
<td>EPY/WPY</td>
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<td>Tarsus length growth</td>
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<td>76.1</td>
<td>0.1427</td>
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**Paternal half-sib comparisons**

<table>
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<th>Variance component</th>
<th>% of total variance</th>
<th>Mean square</th>
<th>d.f.</th>
<th>F</th>
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<td>Residual PHA response</td>
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<td>Body mass growth</td>
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Two-factor analyses of variance, including estimates of variance components, were calculated by the Restricted Maximum Likelihood option in the JMP software (SAS Institute Inc.). Analyses of maternal half-sibs include only broods with both EPY and WPY, those of paternal half-sibs include only offspring of males siring both WPY and EPY. Calculations of growth rates of body mass and tarsus length are described in Methods. Residual PHA responses were calculated from a linear regression of wing-swelling response on body mass for nestlings in all broods. Sample sizes differ between analyses because of losses owing to predation or because the exact hatching time was unknown.

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**Figure 2** Residual wing-swelling response to phytohaemagglutinin (PHA) injection of nestling bluebirds in relation to brood composition of extra-pair (EPY) and within-pair young (WPY). Horizontal lines indicate brood means, black rectangles indicate ±1 s.e., and vertical lines indicate ±1 s.d. In mixed-paternity broods, sample sizes of WPY and EPY differ owing to partial losses of nestlings in either category.
response of the maternal half-sibs of the EPY, that is, the WPY of the cuckolded male, was no higher than that of the WPY of the cuckolder (F_{1.11} = 1.70, P = 0.20). Still, EPY were significantly more immunocompetent than their maternal half-sibs in these broods (F_{1.12} = 4.38, P = 0.041). The fact that males produced more immunocompetent offspring with extra-pair females than with their social mates suggests an interaction effect between male and female genotypes; extra-pair mates have a more favourable combination of genes than social mates.

EPY also had a higher body-mass growth rate than their paternal half-sibs raised in the father’s own nest (Table 1). For this variable, however, the maternal half-sibs of the EPY, that is, the young sired by the cuckolded male in the same nest, also had a significantly higher growth rate than the WPY in the cuckolder’s own nest (F_{1.10} = 22.61, P < 0.0001). Thus, the difference in body-mass growth rate between paternal EPY and WPY (Table 1) was probably due to environmental effects rather than genetic effects. The reason behind this pattern is unknown, but it might be explained by males preferentially seeking extra-pair copulations with females in territories of higher quality than their own or by females investing more in their brood after copulation with particular males.

The immune response in broods of only WPY was similar to that of WPY in broods of mixed paternity (Fig. 2: t = 0.42, P = 0.67). Most females without any EPY may therefore have failed to obtain a better sire for their offspring. Either they may have been constrained in their extra-pair activities, for example as a consequence of male mate guarding, or compatible males may not have been locally available. In bluethroats, extra-pair sires are usually found among the nearest neighbours. The five broods with only EPY did not have a particularly strong immune response, but it was not significantly lower than that of EPY in mixed-paternity broods (F_{5.9} = 1.65, P = 0.11).

All thickness measurements were done twice, and the repeatability was high, ranging between 0.96 and 0.98. Mean values were used in all calculations. All measurements of a particular brood were performed by one person only. The magnitude of the wing-swell response more than doubled from the injection on day 5 to that on day 7 (from 0.43 to 0.93 mm, paired t-test on brood means: t_{8} = 18.26, P < 0.0001). Four broods, which did not receive a sensitizing injection, had a significantly lower swelling response than the rest (t_{7} = 2.23, P = 0.029) and were therefore excluded from between-brood comparisons, but included in within-brood comparisons.

Paternity and molecular sexing

DNA was extracted from blood samples (in a few cases from embryos in unhatched eggs or dead nestlings) using the QIAamp (Qiagen) extraction kit. Paternity was analysed by polymerase chain reaction (PCR) amplification of six microsatellite loci. The combined exclusion probability for these markers was 0.995. The 86 experimental nests contained a total of 300 offspring; 289 with sampled DNA. The offspring were defined as EPY if they showed 0 matches and 2 paternal mismatches (n = 6) but a rather high probability of chance inclusion for the attending male (mean = 0.041 ± 0.038 s.d.) and/or a neighbouring male matching the paternal genotype completely. One offspring showed several mismatches with both parents and was excluded from all analyses (probably an egg contaminant by another female). In summary, EPY amounted to 29% (139 out of 479) of all young and occurred in 59% of all broods. Males were assigned paternity to EPY by the same criteria as for social fathers and WPY above. The mean probability of chance inclusion for EPY with complete match with another male (n = 101) was 0.0052 ± 0.0040 s.d., and for EPY with one mismatch (n = 5) the probability was 0.0023, 0.0021 and 0.0069 in each case. One of the microsatellites, Pocc5, shows Z-chromosome linkage in bluethroats (Aves: Locustinae) and could be sexed according to this locus. To sex the remaining nestlings we used the PCR primers P2 and P8 (ref. 12) which amplify two homologous genes: CHD1W on the Z chromosome and CHD1Z on the Z chromosome. This marker failed to amplify a product in four cases. We also analysed 329 adults (22 males, 19 females) with this marker. In no case did the Pocc5 or the P2/P8 markers disagree with the phenotypic sex determination of adults based on plumage colouration.

Received 24 March, accepted 9 May 2000.

Magnetite defines a vertebrate magnetoreceptor

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The key behavioural, physiological and anatomical components of a magnetite-based magnetic sense have been demonstrated in rainbow trout (Oncorhynchus mykiss). Candidate receptor cells located within a discrete sub-layer of the olfactory lamellae contained iron-rich crystals that were similar in size and shape to magnetite crystals extracted from salmon. Here we show that these crystals, which mapped to individual receptors using confocal and atomic force microscopy, are magnetic, as they are uniquely associated with dipoles detected by magnetic force microscopy. Analysis of their magnetic properties identifies the crystals as single-domain magnetite. In addition, three-dimensional reconstruction of the candidate receptors using confocal and atomic force microscopy imaging confirm that several magnetic crystals are arranged in a chain of about 1 μm within the receptor, and that the receptor is a multi-lobed single cell. These results are consistent with a magnetite-based detection mechanism, as 1-μm chains of single-domain magnetite crystals are highly suitable for the behavioural and physiological responses to magnetic intensity previously reported in the trout.

Permanently magnetized single-domain magnetite crystals (‘lodestone’, Fe-O Fe₃O₄) have been described in many different phyla, and the suitability of magnetite for magnetoreception has been analysed in detail. If the crystals in the nose of the trout are single-domain magnetite, they could act as detector elements within a receptor if their movement, or the torque on them, arising from the interaction of their magnetic moments with the earth’s magnetic field is measurable by the nervous system. Single 50-nm crystals of magnetite do not interact strongly enough with the earth’s magnetic field to overcome the randomizing effects of thermal buffeting; however, if the crystals are arranged in chains as in the magnetotactic bacteria, their individual moments will sum linearly. The average orientation of a freely rotating chain will be aligned with the external field vector, whereas the variance of the chain’s orientation will depend on the intensity of the external field. Chains of crystals with magnetic-to-thermal energy ratios of two and six are optimal for detecting magnetic intensity and direction, respectively. A consistent chain length giving either of the above ratios will thus provide evidence of selection for use of the chains in magnetoreception.

The existence of magnetite-based receptors is difficult to demonstrate because the magnetite crystals are too small to be detected easily and the receptors do not need to be aggregated into a complex sense organ. We previously imaged putative magnetite crystals in thin sections from the nose of the trout but were unable to identify the crystals uniquely nor show that the crystals were organized for use in magnetoreception. To overcome the problems of scale presented by the small size and low volume concentrations of the crystals, we have now combined confocal laser scanning microscopy (CLSM) with atomic force microscopy (AFM)/magnetic force microscopy (MFM) techniques to characterize the putative magnetite crystals and the candidate magnetoreceptor cells in the trout.

Magnetic force microscopy is capable of determining magnetic domain structure in a variety of magnetic materials, including small particles with a spatial resolution of less than 100 nm. Because it is...