Extra-pair paternity among Great Tits *Parus major* following manipulation of male signals


Female Great Tits are known to eavesdrop on the singing behaviour of males. It is unknown, however, whether manipulation of these signals is sufficient to influence extra-pair copulations, or whether such potentially costly reproductive decisions are unaffected by altering short-term signals of condition. Using interactive playbacks, we systematically engaged males in territorial contests in which we could control whether the focal male won or lost the interaction, regardless of the males' true potential. We then determined the levels and patterns of extra-pair paternity among experimental and neighbouring pairs using DNA microsatellite analysis. Extra-pair young were found in equal frequency among the nests of males allowed to win interactions as among those who lost interactions. However, cuckolded males were significantly less variable in allele sizes across the five microsatellite alleles tested than males who were not cuckolded. As measures of genetic variability are increasingly being found to correlate with individual fitness, this may suggest that females are attentive to underlying measures of condition when making extra-pair decisions. Short-term modification of the males' perceived quality may be insufficient to cause females to alter extra-pair decisions.

Extra-pair behaviour is now a commonly described phenomenon among socially monogamous passerines (Petrie and Kempenaers 1998). In many species, extra-pair behaviour is largely female-driven (Smith 1988, Houtman 1992, Kempenaers et al. 1992, 1997, Otter et al. 1998), prompting hypotheses on the benefits females derive from these matings (Kempenaers and Dhondt 1993). Among these is the genetic benefits hypothesis; females seek males higher in quality than the females' mates for the genetic material they can provide the females' offspring (Smith 1988, Kempenaers et al. 1992, 1997, Graves et al. 1993, Weatherhead and Boag 1995, Hasselquist et al. 1996, Otter et al. 1998). However, failure to find correlations between physical characteristics of males and their success in extra-pair paternity has led to other hypotheses being proposed, such as: females assuring fertility of eggs (Wetton and Parkin 1991, Sheldon 1994, Krokene et al. 1998); previewing future mates (Wagner 1991); increasing genetic compatibility or heterozygosity (Brown 1997, Kempenaers et al. 1999, Weatherhead et al. 2000); and gaining material benefits from extra-pair males (Gray 1997).

Great Tits *Parus major* engage in moderate levels of extra-pair fertilizations (Blakey 1994, Krokene et al. 1998, Strohbach et al. 1998, Lubjuhn et al. 1999). Two of these studies, however, failed to find correlations between physical attributes of the males and extra-pair success (Strohbach et al. 1998, Krokene et al. 1998), leading Krokene et al. to suggest that extra-pair behaviour in this species may serve primarily to assure fertilization of the clutch. By contrast, studies on two related species, the Blue Tit *Parus caeruleus* (Kempe-
naers et al. 1997) and the Black-capped Chickadee Poecile atricapilla (Smith 1988, Otter et al. 1998), have shown that females prefer extra-pair males that exceed the females’ mates in some behavioural character – song rates in Blue Tits (Kempenaers et al. 1997) and dominance rank in chickadees (Smith 1988, Otter et al. 1998). Such behavioural characters may also influence choice in female Great Tits, as we recently found that female Great Tits eavesdrop on singing patterns during territorial conflicts among males (Otter et al. 1999).

Otter et al. (1999) used interactive playback to initiate territorial disputes with resident Great Tit males in a Danish population. By controlling the degree to which our playback songs overlapped the songs of the resident male, we controlled whether the male “won” or “lost” these interactions. Females mated to males who lost interactions against our playbacks were more likely to intrude into neighbouring territories than were females whose mates won interactions. Moreover, intruding females preferentially intruded into the territories of males who had fared well against our playbacks, demonstrating that females were eavesdropping on these territorial interactions. It is unknown, however, whether these intrusions lead to extra-pair behaviour or represented surreptitious approaches by females to further assess males. Such covert singing is common during the mating period (see Bensch and Hasselquist 1992), and appears to be used by females in some species during post-mating assessment (Neudorf et al. 1997).

In this study, we assess the paternity in the nests of the experimental pairs used in the study of Otter et al. (1999) and neighbouring non-experimental pairs. Our aim was to determine whether short-term experimental manipulation influences extra-pair matings as well as intrusions. Using DNA microsatellites to assess parentage, we determined the extent and patterns of extra-pair paternity in relation to male roles in playback experiments.

We also measured extra-pair success against an index of microsatellite heterozygosity within and among individuals developed by Coulson et al. (1998) – the d2 index. The d2 index of variability in allele sizes allowed us to test an alternate hypothesis of extra-pair behaviour – genetic compatibility of mates – as well as provided an alternate method of testing the genetic benefits hypothesis. Heterozygosity, especially in microsatellites, is often correlated with measures of fitness (Allendorf and Leary 1986, Coulson et al. 1998, David 1998) suggesting that this index may represent a means of testing underlying fitness of males independent of their role in playbacks.

Methods

The study was conducted February through June 1998 at the Strodam Biological Reserve 40 km north of Copenhagen, Denmark. The study area consisted of 80 ha of mature open beech forest inhabited by 35 to 40 pairs of Great Tits each year. Adult birds were captured using a combination of mistnets and potter traps at winter feeders or by catching breeding birds in nestboxes during the spring. At the time of capture, birds were given a unique combination of coloured legbands, sexed by ventral breast stripe width and aged by coloration of the primary coverts relative to the primaries (see Gosler 1993). A 75 µl blood sample was taken from the wing by brachial puncture at the time of banding, and then stored in 1 ml of Lysis buffer at 4°C until DNA was extracted.

Beginning on 1 April, each area of the study site was visited every second day to map the territories of the resident birds and determine which nestboxes were used for breeding. Playbacks were initiated once females had fully lined nests up to the first days of egg laying (20–30 April), coinciding with the peak fertile period.

Playbacks

Design of the playbacks has been reported in detail elsewhere (Otter et al. 1999). Two neighbouring males constituted a single dyad; nine dyads received playbacks during the course of the study (N = 18 males in total). The two males in each dyad were recorded during normal daytime singing to determine the composition and size of their repertoires. A shared song type was chosen as the playback stimulus. Playbacks were conducted over two successive days. The male in the dyad that received the first playback was chosen randomly, and the order of playbacks was reversed on the second day. Each male, however, received the same treatment on both days of playback.

One male was randomly designated to receive an escalating playback, in which we overlapped the singing behaviour of the male and played back songs with longer strophe lengths than that sung by the focal male. If the male stopped responding during the 3-min playback, we played a song with the same strophe length as our last stimulus every ten seconds until the male either resumed singing (in which case we resumed the playback response) or the trial ended. To any eavesdropping female, this treatment would represent a resident male having difficulty evicting a “stranger” from his territory.

After the first playback ended, we immediately set up the system in the territory of the dyadic counterpart and initiated the second male’s playback. The second male received a de-escalating playback, in which we alternated singing with the male, played back song with shorter strophe lengths than the male sang and after two minutes responded only to every second song of the male. If the male stopped singing, we did not reinitiate playbacks until the trial had either ended or

the male resumed singing (in which case we resumed the playback treatment). This treatment may be perceived by eavesdroppers as the second male being better able to evict the “stranger” that had previously frustrated the neighbour.

**Breeding behaviour and nestling sampling**

Nestboxes used by pairs were checked every 2 days from 1 April to initiation of incubation. This allowed us to determine date of first egg and total clutch size. Once incubation had begun, nests were monitored visually for continued activity, and rechecked on day 13, and every 2 days thereafter to determine date of hatch. At 7 days after hatching, a 30–50 µl blood sample was collected from each nestling by brachial puncture.

Of the males used in the original playback studies, the nests of 13 were sampled for paternity (6 escalation, and 7 de-escalation treatments). The remaining five pairs used in the playback studies either did not initiate nests in nestboxes within their territories and no natural cavity nests were located (N=4) or nests were depredated (N=1). In addition, 10 nests from neighbouring pairs were also sampled to determine whether females from neighbouring territories sought out playback males as extra-pair sires.

**Paternity analysis**

The microsatellite markers used in this study were developed initially for work on other species (see references in Appendix 1), but were also found to be highly polymorphic in Great Tits. The basic PCR protocol for markers is described here, with specific reactions for each primer given in Appendix 1.

DNA was extracted from approximately 1 µl of blood/buffer mix using a chelating resin (“Chelex”, Walsh 1991). Each 15 µl reaction mix included approximately 10 ng of genomic DNA with 0.1 units of Taq polymerase (Applied Biotechnologies), 0.8 µM of each primer, and 10 µl of Jeffreys’ buffer (final concentration: 20 mM (NH₄)₂SO₄, 75 mM Tris-HCl pH 8.8, 0.15 mM DNA-ase free BSA, 10 mM β-mercaptoethanol, 1.0 mM MgCl₂; Jeffreys et al. 1988).

Cycling at the loci Pdo5 and Pocc6 was performed using a Hybaid thermal cycler, whereas cycling at the remainder of the loci was performed using a Perkin-Elmer model 480. Products were resolved on a 4% denaturing polyacrylamide gel and were visualized by silver staining (Bassam et al. 1991).

Broods were run alongside their social parents, and alleles were sized with reference to a 10 base-pair ladder (Gibco BRL) run in at least two lanes on each gel. Nestlings were designated as extra-pair if they mismatched their putative father at two or more loci. All nestlings were scored without reference to the playback treatment of their putative fathers.

The sequences for the five microsatellite primers used in this study are reported in Appendix 1. The heterozygosity and variability at each locus were assessed using 21 presumably unrelated adults, and these data were used to generate the probability of excluding a nestling from its putative parents (Gundel and Reetz 1981) (Table 1). The probability of excluding parentage from mismatched nestlings using this array of five loci was 0.9992.

**Heterozygosity indices (d²)**

Indices of genetic heterozygosity within and between individuals were calculated by the d² values (Coulson et al. 1998). This technique calculates the difference in size between two alleles within the same individual, or between two individuals, and thus quantifies the degree of heterozygosity in microsatellite markers. Assuming that small differences in allelic size may indicate much more recent mutation events, Coulson et al.’s index may indicate that less heterozygous individuals have experienced more recent periods of inbreeding than more heterozygous individuals. This index can also indicate a potentially closer relationship between two individuals with similar-sized alleles.

d² is calculated as the squared difference between two alleles for a particular locus, divided by the number of base pairs making up the repeat unit of the microsatellite (two for all dinucleotide primers used here). The d² scores for an individual are then averaged for all loci (see Coulson et al. 1998). To calculate the degree of the size difference in microsatellite alleles between males and females in a pair, the four pair-wise comparisons of allele sizes between the two individuals were averaged for each locus (i.e. male allele 1 vs female allele 2, male allele 2 vs female allele 1, etc.). This score was averaged over all five loci to obtain a d² value between two individuals within a pair.

Table 1. Probability of exclusion using the five microsatellite markers.

<table>
<thead>
<tr>
<th>Locus</th>
<th>No. of alleles</th>
<th>Observed heterozygosity</th>
<th>P(ex)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pdo5</td>
<td>8</td>
<td>0.91</td>
<td>0.68</td>
</tr>
<tr>
<td>Pocc6</td>
<td>12</td>
<td>0.88</td>
<td>0.74</td>
</tr>
<tr>
<td>AFC04466 (BT6)</td>
<td>7</td>
<td>0.71</td>
<td>0.63</td>
</tr>
<tr>
<td>Pca8</td>
<td>13</td>
<td>0.92</td>
<td>0.80</td>
</tr>
<tr>
<td>Ppi2</td>
<td>19</td>
<td>0.88</td>
<td>0.87</td>
</tr>
<tr>
<td>Combined P(ex)</td>
<td></td>
<td></td>
<td>0.9992</td>
</tr>
</tbody>
</table>

DNA profiles supported by the fact that in nests where both parental males were identiﬁed. We maintained a conservative approach in these nests, assigning mixed parentage only where banding patterns indicated no possibility of parentage being by a single male or female.

Nine of 23 nests had mixed paternity (39.1%). The proportion was similar between nests where blood samples were collected from both social parents (6/17 nests) and where mixed parentage was deduced by allelic patterns across the ﬁve probes. Mixed paternity was assumed if a brood contained three or more alleles at a single locus that could not be attributed to the female. Since one male can only contribute two alleles at any locus, any excess alleles must represent mixed paternity of the brood – such mixed paternity was conﬁrmed where EP males were identiﬁed. We maintained a conservative approach in these nests, assigning mixed parentage only where banding patterns indicated no possibility of parentage being by a single male or female.

Paternity in relation to playbacks

Neither of the two playback-treatment nests which lacked a blood sample from the male showed indications of mixed paternity. We conservatively assigned these nestlings to the putative father. This decision is supported by the fact that in nests where both parental DNA proﬁles were known, EPY were predominately in minority of the clutch suggesting that full cuckoldry is a rare event.

We found no effect of the playback on probability of EPY in nests. Three de-escalation treatment males had EPY in their clutches versus two escalation treatment males. In fact, within those nests, de-escalation males tended towards a higher proportion of the clutch that was extra-pair (0.36 vs 0.11) although the small sample sizes preclude statistical analysis.

The identity of the EP male was determined in only three of the experimental nests which contained EPY; the remaining two nests were on the edge of the study area and extra-pair males were presumably un-banded males outside the reserve. A single neighbouring male sired the offspring in two cases, while one nest had young sired by two neighbouring males (one sired 3, the other a single nestling of the 10 in the brood). Two females chose an extra-pair male who had been an experimental “winner” (de-escalation treatment), one chose an experimental “loser”.

Microsatellite variability and paternity

Variability of allele sizes was determined among the 17 nests with both male and female blood samples using the d^2 index. Males that were cuckolded (N = 6) had lower d^2 values than males who were not cuckolded (N = 11) (t-test, assuming unequal variance: t = 2.67, df = 15, P = 0.02) (Table 2). By comparison, there was no signiﬁcant difference in d^2 values of females with or without EPY (t = −0.53, df = 15, P = 0.61). There was also no signiﬁcant difference in variability of allele sizes between the male and female in pairs where extra-pair young did and did not occur (t = 0.43, df = 15, P = 0.68).

Discussion

Playback treatments did not appear to inﬂuence extra-pair paternity in the same manner they inﬂuenced intrusion rates found by Otter et al. (1999). We found females with extra-pair young to be almost equally distributed between the two treatment types. Assigning paternity to EPY added little to determining female decisions; females chose extra-pair males from our playback study in only three cases, with two EP males from

Table 2. Measures of allelic variability (d^2) across microsatellite loci in relation to presence of extra-pair young in a male’s own brood.

<table>
<thead>
<tr>
<th></th>
<th>Extra-pair young in own brood</th>
<th>No extra-pair young in own brood</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>45.2 ± 12.0</td>
<td>152.7 ± 28.1</td>
<td>0.02</td>
</tr>
<tr>
<td>Female</td>
<td>233.3 ± 102.9</td>
<td>137.1 ± 42.3</td>
<td>NS</td>
</tr>
<tr>
<td>Pair</td>
<td>100.6 ± 23.2</td>
<td>119.4 ± 16.7</td>
<td>NS</td>
</tr>
</tbody>
</table>

* P-values from t-test with unequal variance between groups.

Results

Level of EPY of offspring

Twenty-three nests were sampled for paternity, in 17 of which blood samples were obtained from both male and female at the nest. In four nests, blood samples were obtained from the female only and in two from the male only. Only two of the experimental nests (males receiving playbacks) did not have blood samples from the putative father, the remaining nests missing one parental blood sample occurred among pairs neighbouring experimental nests. In these latter nests, paternity/maternity was determined by allelic patterns across the ﬁve probes. Mixed paternity was assumed if a brood contained three or more alleles at a single locus that could not be attributed to the female. Since one male can only contribute two alleles at any locus, any excess alleles must represent mixed paternity of the brood – such mixed paternity was conﬁrmed where EP males were identiﬁed.
the de-escalation treatments and one from escalation treatments.

Males that are less variable in microsatellite loci were, however, more likely to have extra-pair young within their nests than males with higher d² values. This trend was not seen in females, and neither were pairs with similar levels of variability among alleles likely to have more extra-pair young, as predicted by genetic compatibility models of extra-pair behaviour (Brown 1997, Kempenaers et al. 1999). Heterozygosity has been found to correlate with survival potential in red deer (Cervus elaphus) and both juvenile growth-rates and parental egg size in chinook salmon (Oncorhynchus tshawytscha) (D. D. Heath, C. A. Bryden, J. M. Shrimpton, G. K. Iwama, J. Kelly and J. W. Heath, unpublished) suggesting that this index may be indicative of general vigour of animals. While increasing heterozygosity is generally considered an indicator of underlying fitness of the organism (Allendorf and Leary 1986), the possibility of a d²–fitness correlation requires further investigation in Great Tits. Microsatellites may have certain advantages for assessing variability–fitness correlations (Coulson et al. 1998, Luikart and England 1999), as non-coding microsatellites and have a higher potential to accumulate mutations than allozymes. Furthermore, if length polymorphisms accumulate by single addition/deletions of microsatellite units, the degree of variability may serve as a fine-scale molecular clock since periods of past inbreeding (Luikart and England 1999). Comparisons of microsatellite variability with condition-dependent plumage badges (Norris 1993) or singing behaviour in Great Tits may further clarify the importance of heterozygosity as an index of condition in this species.

Our results suggest that playbacks may have induced females to intrude to reassess their judgements of relative male quality, but a female’s final decision whether to engage in extra-pair copulations was made on real differences between males rather than those indicated by our playbacks. The intrusions we reported in Otter et al. (1999) never led to any observed extra-pair copulations, leading us to suggest that approaches may have been for the purpose of assessment. As males were assigned randomly to the two playback treatments, it is highly likely that the roles we indicated for males commonly contradicted a female’s previous assessment of her mate and neighbouring males. Intrusions may have been attempts by females to increase the certainty of their original assessments, but females stopped short of soliciting a potentially costly EPC. Slagsvold and Lifjeld (1997) have argued that females may forgo extra-pair copulations when they lack sufficient information to accurately judge the quality of their mate relative to available alternative males. It may, therefore, be common for females to continually monitor males to maintain and update their assessments of them. Our experimental playbacks may have prompted the periods of re-assessment that we witnessed. However, the costs associated with extra-pair copulation with males assessed during a short period of playbacks may be too large to override the females’ original assessments of relative quality between males.

Recent studies on Great Tits (Krokene et al. 1998, Strohbach et al. 1998, Lubjuhn et al. 1999) failed to find support for females basing extra-pair decisions on physical attributes of males. Our study suggests that females may not base decisions on short-term alteration of behavioural cues. However, our work does indicate that the degree of allelic variability in microsatellites of males may influence their probability of having extra-pair young in their brood. We found, however, little evidence for the idea that females engaged in EPCs based on genetic similarity between themselves and their mate, although our sample sizes were small. The phenotypic cues that females use in the acceptance or solicitation of extra-pair partners remain uncertain. Further studies should measure natural variation in behavioural cues, such as song rates during the dawn chorus or fighting propensity during natural interactions, in order to determine the connection between assessable traits and the allelic variability in microsatellites. If allelic variability is correlated with genetic health of the organism, this should be expressed in condition-dependent cues that females could use for assessment purposes.

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References


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Appendix 1. Primer Sequences and PCR protocols for the microsatellite markers.

BT6 (Full name AFO41466) – S. M. Tanner, H. Richner and D. Schuemperli (unpublished)

Primer sequence
F: CTT CCT GCA GTT GCC TCC CG
R: GTG GGC CAT GTT TAT AGC CTG GCA CTA
AGA AC

PCR conditions
95°C 2 min; 30 cycles of 95°C 1 min, 55°C 1 min, 72°C 2 min; final cycle 72°C 7 min

Pea8 – Dawson et al. (2000)

Primer sequence
F: ACT TCT GAA ACA AAG ATG AAA TCA
R: TGC CAT CAG TGT CAA ACC TG

PCR conditions
94°C 2 min; 35 cycles of 94°C 20 s, 56°C 30 s, 72°C 1 min

Pdo5 – Griffith et al. (1999)

Primer sequence
F: GAT GTT GCA GTG ACC TCT CTT G
R: GCT GTG TTA ATG CTA TGA AAA TGG

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PCR conditions
94°C 2 min; 35 cycles of 94°C 20 s, 52°C 30 s, 72°C 1 min

*P. occidentalis* – Beash et al. (1997)
Primer sequence
F: TCA CCC TCA AAA AAC ACA CAC ACA
R: ACT TCT CTC TGA AAA GGG GAG C

PCR conditions
94°C 2 min; 35 cycles of 94°C 20 s, 56°C 30 s, 72°C 1 min

*P. pi/2* – Martinez et al. (1999)
Primer sequence
F: CAC AGA CCA TTC GAA GCA GA
R: GCT CCG ATG GTG AAT GAA GT
PCR conditions
94°C 2 min; 35 cycles of 94°C 20 s, 56°C 30 s, 72°C 1 min