No effect of parental quality or extrapair paternity on brood sex ratio in the blue tit (Parus caeruleus)

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Sex allocation theory predicts that parents should manipulate brood sex ratio in order to maximise the combined reproductive value of their progeny. Females mating with high quality males should, therefore, be expected to produce brood sex ratios biased towards sons, as male offspring would receive a relatively greater advantage from inheritance of their father’s characteristics than would their female siblings. Furthermore, it has been suggested that sex allocation in chicks fathered through extrapair fertilizations should also be biased towards sons. Contrary to these predictions, we found no evidence that the distribution of sex ratios in a sample of 1483 chicks from 154 broods of blue tits (Parus caeruleus) deviated significantly from that of a binomial distribution around an even sex ratio. In addition, we found no significant effect on brood sex ratio of the individual quality of either parent as indicated by their biometrics, feather mite loads, time of breeding, or parental survival. This suggests that females in our population were either unable to manipulate offspring sex allocation or did not do so because selection pressures were not strong enough to produce a significant shift away from random sex allocation. The paternity of 986 chicks from 103 broods was determined using DNA microsatellite typing. Extrapair males sired 115 chicks (11.7%) from 41 broods (39.8%). There was no significant effect of paternity (within-pair versus extrapair) on the sex of individual offspring. We suggest that, in addition to the weakness of selection pressures, the possible mechanisms responsible for the allocation of sex may not be sufficiently accurate to control offspring sex at the level of the individual egg. Key words: Blue tit, DNA microsatellite typing, extrapair paternity, parental quality, Parus caeruleus, sex ratio. [Behav Ecol 12:674–680 (2001)]
dicted to be of higher relative quality in comparison to a female’s social partner (Otter et al., 1998; Whittingham and Lifjeld, 1995; but see Stutchbury et al., 1997). We would consequently predict a male-biased sex ratio amongst extrapair chicks. However, no such relationship has been found in the few recent studies in birds (Saïno et al., 1999; Sheldon and Ellegren, 1996; Westerdalh et al., 1997), although some preliminary data for blue tits suggests that extrapair chicks are more likely to be male (Kempenaers et al., 1997).

The aim of this study was to investigate the effects of parental quality and offspring paternity on brood sex ratios in the blue tit. Parental quality was assessed using biometric measurements and survival to subsequent breeding seasons, while paternity was determined using DNA microsatellite markers.

**METHODS**

**Field measurements**

Data were collected during the breeding seasons of 1997 to 1999 from box nesting blue tits in six separate, small (< 30 ha) deciduous and mixed woods in northwest Lancashire, U.K. Adult birds were caught at feeding stations during the winter using mist nets or at the nest while provisioning nestlings. The majority of adult birds were individually marked with a numbered metal ring and a combination of three color rings so that they could be identified in the field.

Body size, ectoparasite load, extent of post-juvenile molt and over-winter survival were identified as potential indicators of individual quality. Body size may influence the ability to compete for food (Garnett, 1981) and obtain a breeding territory (Drent, 1983). Right wing length (maximum chord to nearest mm), right tarsus length (to nearest 0.1mm) and mass (to nearest 0.1g) were recorded using a fixed rule, calipers, and a spring balance respectively. Repeatability (r) values for tarsus and wing length were calculated according to the method in Lesells and Boag (1987), and were based on data collected by three observers, with individual birds measured at approximately annual intervals. Tarsus length was significantly, moderately repeatable (r = .42). 

Molt scores for adult birds (more than 1 year old) are scored as the number of juvenal greater secondary coverts lost (Behnke et al., 1999; Wiles et al., 2000).

**Molecular methods**

**Sexing techniques**

DNA was made available for amplification either by a standard protease-K/ phenol chloroform extraction (Kawasaki, 1990) or through a Chelex® resin-based technique (Walsh et al., 1991). Sex was determined by polymerase chain reaction (PCR) amplification of the CHD1-W and CHD1-Z genes using the primers P2 and P8 (Griffiths et al., 1998). Products amplified from the 1997 and 1998 samples were separated by electrophoresis through 6% denaturing polyacylamide gels and visualized by silver staining (Bassam et al., 1991). In 1999, products were separated in 2.5% agarose gels containing ethidium bromide and visualized under UV light. The CHD1-W gene is on the W chromosome and CHD1-Z on the Z chromosome. In birds, the heterogametic female possesses both genes, while the male possesses only CHD1-Z. In blue tits the product amplified from the CHD1-W gene is 25 base pairs larger than the product for the CHD1-Z gene and, therefore, a female sample exhibits two clear bands in contrast to the single band of a male sample. To validate our procedures, PCR products for 172 birds of known gender (from behavioral observations) were blindly scored for sex. These results agreed with the field sexings in 99.4% (171/172) of cases. The one disagreement was probably the result of a mistake in the field when reading rings or in the laboratory with tube labeling.

**Paternity analysis**

Paternity was analyzed using PCR amplification of 11 polymorphic DNA microsatellite loci (Table 1). Novel loci that increased the power of the analysis became available as the project proceeded and, accordingly, the set used varied between subsets of the data. Products were separated in 6% polyacylamide gels and visualized using silver staining (Bassam et al., 1991). Allele sizes were calculated by direct comparison of PCR products for 172 birds of known gender (from behavioral observations). The accuracy of assignment is measured by the exclusion probability of each array, where exclusion probability is defined as the likelihood of a nestling being correctly assigned paternity if its genotype matches that of the putative father at all loci used in the array. All arrays used had a minimum exclusion probability of 0.976 as calculated by a maximum likelihood procedure using the CERVUS computer program (Marshall et al., 1998) (Array A = 0.976, B = 0.985, C = 0.997, D = 0.996; Table 1). Offspring mismatching the putative father at any locus were assigned as resulting from extrapair or within-pair fertilizations according to the maximum likelihood procedure used in the CERVUS computer program (Marshall et al., 1998), where the confidence level for assignment was set at a minimum of 80%. The program calculates the likelihood that a putative male is the true father.
Table 1
Microsatellite markers used in the paternity analysis

<table>
<thead>
<tr>
<th>Locus</th>
<th>No. alleles</th>
<th>Observed heterozygosity</th>
<th>Minimum exclusion probability</th>
<th>Set code</th>
<th>EMBL accession no.</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PK11</td>
<td>7</td>
<td>0.844</td>
<td>0.380</td>
<td>A</td>
<td>AF041465</td>
<td>Tanner et al. (personal communication)</td>
</tr>
<tr>
<td>PK12</td>
<td>16</td>
<td>0.875</td>
<td>0.573</td>
<td>A,C,D</td>
<td>AF041466</td>
<td>Tanner et al. (personal communication)</td>
</tr>
<tr>
<td>Axl18</td>
<td>16</td>
<td>0.831</td>
<td>0.647</td>
<td>A,D</td>
<td>AF276575</td>
<td>Richardson et al. (in press)</td>
</tr>
<tr>
<td>My4</td>
<td>19</td>
<td>0.938</td>
<td>0.759</td>
<td>B</td>
<td>U82388</td>
<td>Double et al., 1997</td>
</tr>
<tr>
<td>PAT MP2–43</td>
<td>5</td>
<td>0.750</td>
<td>0.288</td>
<td>B</td>
<td></td>
<td>Otter et al., 1998</td>
</tr>
<tr>
<td>Pov2</td>
<td>9</td>
<td>0.656</td>
<td>0.372</td>
<td>A</td>
<td>AJ279804</td>
<td>Dawson et al., 2000</td>
</tr>
<tr>
<td>Pov3</td>
<td>20</td>
<td>0.909</td>
<td>0.656</td>
<td>C,D</td>
<td>AJ279805</td>
<td>Dawson et al., 2000</td>
</tr>
<tr>
<td>Pov7</td>
<td>13</td>
<td>0.688</td>
<td>0.620</td>
<td>B</td>
<td>AJ279809</td>
<td>Dawson et al., 2000</td>
</tr>
<tr>
<td>Pov8</td>
<td>33</td>
<td>0.922</td>
<td>0.818</td>
<td>A,C,D</td>
<td>AJ279810</td>
<td>Dawson et al., 2000</td>
</tr>
<tr>
<td>Pds6</td>
<td>20</td>
<td>0.938</td>
<td>0.776</td>
<td>B,C</td>
<td>Y15125</td>
<td>Griffith et al., 1999</td>
</tr>
<tr>
<td>Poc6</td>
<td>20</td>
<td>0.877</td>
<td>0.620</td>
<td>C,D</td>
<td>U59117</td>
<td>Bensch et al., 1997</td>
</tr>
</tbody>
</table>

Four alternative sets of loci were used (A–D). Expected heterozygosity and minimum exclusion probability values were calculated using a marker analysis in CERVUS (Marshall, 1998). Minimum exclusion probability values represent the exclusion probability assuming the genotype of only one parent is known.

* CERVUS.

given the observed genotypes and their relative frequency in the population (Marshall et al., 1998).

Statistical methods
Brood sexes are expressed as the proportion of sexed individuals that were male. Analyses were performed in S-Plus 2000 and follow the methods used by Westerdahl et al. (1997). For the main analysis of brood sex ratio and offspring sex, we used generalized linear models (GLM) with binomial errors and a logit link (McCullagh and Nelder, 1989; Venables and Ripley, 1999). The statistical significance of various terms in the model is determined by calculating the deviance of the model with and without those terms, where the deviance is distributed approximately as \( \chi^2 \) (Crawley, 1993). To test the overall sex ratio distribution for departure from a binomial distribution we use a robust randomization method which incorporates an element to account for variable brood sizes (Westerdahl et al., 1997).

In the analysis of sex ratio in relation to parental characteristics we use the brood as the unit of analysis and the number of chicks sexed as the binomial denominator. In the analysis of offspring sex in relation to paternity we use the chick as the unit of analysis but enter a term as a nest-box identifier into the model to control for any effects of shared origin. Additionally, we allowed for any over-dispersion that this may cause by setting the dispersion parameter according to the variance of the data (McCullagh and Nelder, 1989) although in practice, for both models, the dispersion parameter was approximately one, as expected for binomial data. Power analysis was carried out for nonsignificant (ns) results and follows the procedures in Cohen (1976) and the G^*Power computer program (Faul and Erdfelder, 1992).

RESULTS
Brood sex ratio and parental quality
During the three study years, the sexes of 1483 chicks from 154 broods were determined, of which 712 were male. The mean brood sex ratio was 0.48 (range = 0.00–0.88, SD = 0.16) (Figure 1). The number of sons and daughters from each brood did not differ significantly (paired \( t \) test, \( t = 1.83, df = 153, p = .069 \)), although there was a tendency for a skew towards daughters. The power of these \( t \) tests was relatively high (Power = 92% for a medium to small effect size of \( d = 0.4 \)). We were unable to sex 146 individuals (9.0% of the total number of eggs laid) due to either eggs being infertile (\( N = 72 \)), early hatching mortality, or failure to obtain PCR products, and, therefore, the values given above may differ from those for the primary sex ratio. There was no significant difference between the mean sex ratio of completely and incompletely sexed broods (two sample \( t \) test, \( t = 0.26, df = 152, p = .98 \)). This does not, however, necessarily suggest that our sex ratio values reflected the primary sex ratio before any differential mortality of sons and daughters had occurred prior to sample collection (Fiala, 1981).

The distribution of brood sex ratios did not differ significantly from a binomial distribution (\( n = 154, p = .90, 65\% \) power of detecting a skew as small as 0.60 sons to 0.40 daughters). A similar result was found even if unsexed offspring were presumed to be either all male or all female and sex ratios were recalculated accordingly (maximum male skew, \( p = .12 \); maximum female skew, \( p = .10 \)). This result suggests that the female blue tits in our study did not manipulate brood sex ratio away from a random allocation between the
The model is binomial with a logit link; dispersion parameter (variance) = 1.10. Interactions of “Year” and “Wood” with the other main effects were all nonsignificant (p > .1) and were dropped from the final model. All main effects that were tested are included in the table, although none significantly influenced the response variable. The deviances shown are the changes in deviance when the term is removed from the final model. The power of this analysis is 80% for a medium effect size of w = 0.3, where df = 1.

Table 2
Analysis of deviance table resulting from a Generalized Linear Model with number of sons as the response variable and number of chicks sexed as the binomial denominator

<table>
<thead>
<tr>
<th>Term</th>
<th>df</th>
<th>Change in deviance</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Null model</td>
<td>91</td>
<td>95.48</td>
<td>ns</td>
</tr>
<tr>
<td>Year</td>
<td>2</td>
<td>2.09</td>
<td>ns</td>
</tr>
<tr>
<td>Wood</td>
<td>4</td>
<td>2.67</td>
<td>ns</td>
</tr>
<tr>
<td>Male age</td>
<td>1</td>
<td>0.05</td>
<td>ns</td>
</tr>
<tr>
<td>Male wing length</td>
<td>1</td>
<td>0.16</td>
<td>ns</td>
</tr>
<tr>
<td>Male weight</td>
<td>1</td>
<td>0.07</td>
<td>ns</td>
</tr>
<tr>
<td>Male tarsus length</td>
<td>1</td>
<td>0.79</td>
<td>ns</td>
</tr>
<tr>
<td>Male mite score</td>
<td>1</td>
<td>0.04</td>
<td>ns</td>
</tr>
<tr>
<td>Male survival</td>
<td>1</td>
<td>0.78</td>
<td>ns</td>
</tr>
<tr>
<td>Female age</td>
<td>1</td>
<td>0.37</td>
<td>ns</td>
</tr>
<tr>
<td>Female wing length</td>
<td>1</td>
<td>0.67</td>
<td>ns</td>
</tr>
<tr>
<td>Female weight</td>
<td>1</td>
<td>2.98</td>
<td>ns</td>
</tr>
<tr>
<td>Female tarsus length</td>
<td>1</td>
<td>0.0005</td>
<td>ns</td>
</tr>
<tr>
<td>Female mite score</td>
<td>1</td>
<td>0.36</td>
<td>ns</td>
</tr>
<tr>
<td>Female survival</td>
<td>1</td>
<td>2.56</td>
<td>ns</td>
</tr>
<tr>
<td>First egg date</td>
<td>1</td>
<td>1.30</td>
<td>ns</td>
</tr>
<tr>
<td>Clutch size</td>
<td>1</td>
<td>0.19</td>
<td>ns</td>
</tr>
<tr>
<td>Number of fledglings</td>
<td>1</td>
<td>0.84</td>
<td>ns</td>
</tr>
</tbody>
</table>

The model family is binomial with a logit link; dispersion parameter (variance) = 1.11. The term “Brood” is included to link offspring raised in the same nests. The deviances shown are the changes in deviance when the term is removed from the final model. The power of this analysis is 80% for a medium effect size of w = 0.3, where df = 1.

The model family is binomial with a logit link; dispersion parameter (variance) = 1.11. The term “Brood” is included to link offspring raised in the same nests. The deviances shown are the changes in deviance when the term is removed from the final model. The power of this analysis is 80% for a medium effect size of w = 0.3, where df = 1.

Offspring sex and paternity
We could check the putative parentage of 986 chicks from 103 broods. Using CERVUS (Marshall et al., 1998; Slate et al., 2000), we could exclude the putative father as the most likely genetic father for 115 offspring (11.7% of chicks from 41 [39.8%] broods) and this exclusion likelihood was statistically significant for 89 of these offspring. Here, we treat all 115 offspring as being the result of extrapair fertilizations. If we only treat as extrapair those 89 offspring for which paternity was significantly more likely to be due to a nonpatrilineal father, the conclusions of the following analysis remain the same. We were unable to determine paternity for a further 49 offspring/eggs from these broods (4.97% of the total number of offspring) due to mortality prior to sampling or failure to obtain PCR products. The distribution of extrapair chicks among broods was significantly different from a binomial distribution (calculated as above and Westerdahl, 1997 n = 95, p < .0001). If we conservatively take the two extreme cases, in which all chicks for which paternity had not been determined were assigned either as within-pair, or as extrapair, and the test was repeated in each case, the result remained the same. A repeatability analysis for extrapair fertilizations containing both completely within-pair and mixed paternity broods showed a significant, moderate repeatability for individual males between years (n = 12, r = .59, F(1, 12) = 4.79, p = .006), suggesting that some individuals are consistently susceptible to cuckoldry regardless of their age. A similar test could not be performed for females due to the small sample size.

A comparison of extrapair offspring frequency with sex ratio across broods, as suggested by Westneat et al. (1995), provides one test for a positive correlation between sex ratio and level of extrapair paternity. However, as noted by Sheldon and Ellegren (1996), if females mated to high quality males are biasing their brood sex ratio towards sons whilst not actively pursuing extrapair copulations, a negative correlation is predicted. The effect of paternity on offspring sex was therefore analyzed at the level of the individual chick using a GLM, controlling for the effects of common brood (see Statistical Methods section). The results show that neither the paternity of the chick (within-pair or extrapair father) nor the paternity of the brood (mixed paternity or single father) had any significant effect on the sex of offspring (Table 3), which implies that female blue tits in our study were not assigning individual chicks to a particular sex on the basis of paternity.

The significant effect of the interaction between brood and paternity indicates that the size of the difference between within-pair and extrapair sex ratios varies significantly between some broods. To investigate this further, we calculated the sex ratio difference for each brood (within-pair chicks’ sex ratio minus extrapair chicks’ sex ratio), and compared this between years and breeding woods. There was no significant effect of year or wood on sex ratio difference, although there was a trend towards an effect of breeding wood (two-way ANOVA,
populations may be an important factor (Dale et al., 1999; Griffith et al., 1999). A female-biased operational sex ratio would result in decreased selection pressure on traits used by females to determine male quality, reducing the reliability of the signal and thus a female’s ability to assess male status and adjust the brood sex ratio accordingly. However, the nonrandom occurrence of extrapair fertilizations in our population suggests that variation in female assessment of male quality should occur. Variability of environmental selection pressures could also explain the between-population variance in correlates of sex ratio. An alternative hypothesis suggests that females adjust brood sex ratio in relation to territory quality (Richner et al., 1993) which may be positively correlated with male quality. We have no measure of territory quality in our study, but if it shows little variance then the selection pressure for adaptive sex ratio manipulation might be low. We found no significant effect of fledging success, independently of clutch size, on brood sex ratio, indirectly suggesting that territory quality, or factors that influence nest productivity did not influence the brood sex ratio.

**Female quality and brood sex ratio**

The maternal condition hypothesis (Trivers and Willard, 1973) predicts that females should adjust their brood sex ratio according to their own condition when eggs are laid, although male quality may be a confounding variable in populations where assortative mating occurs, as in the blue tit (Andersson et al., 1998). A study on lesser black-backed gulls, *Larus fuscus*, provides experimental evidence supporting this theory; females in better body condition tended to have more sons (Nager et al., 1999). However, we found no significant relationship between any of our measures of female quality and brood sex ratio, a similar result to that found in several other observational studies on passerines (e.g., Bradbury et al., 1997; Kolliker et al., 1999; Westerdahl et al., 1997; but see Westerdahl et al., 2000). These results indicate either that our measures do not provide an accurate indication of female condition, or that variation in female quality is not sufficient to instigate a detectable level of variance among sex ratios between broods in our population.

**Paternity and brood sex ratio**

The distribution of extrapair paternity among broods in our population differed significantly from that expected due to random allocation, suggesting that there was significant variation in the probability of a male’s nest containing chicks fathered through extrapair copulation. There was, however, no significant relationship between the sex of a chick and its paternity, which might be expected if females were able to manipulate the sex allocation adaptively to individual chicks. This result matches the findings of most previous studies, both observational (Sheldon and Ellegren, 1996; Westerdahl et al., 1997) and experimental (Saino et al., 1999), although it differs from the preliminary results found in another study of blue tits, where there was a suggestion that extrapair offspring were more likely to be male (Kempenaers et al., 1997).

We may not have measured the trait that is important in determining the incidence of extrapair paternity in our population. Preliminary analysis suggests that the proportion of extrapair nestlings in a brood is unrelated to the male nest holder’s biometrics or breeding age, although there was a significant effect with the wood in which the birds bred, suggesting that habitat features may be important in determining the incidence of extrapair paternity (unpublished data). Despite this, there was no effect of the breeding wood on sex ratio in the above analyses.

Although we found no evidence for selection on sex allocation, the mechanisms of the procedure may not be accurate
enough to target specific eggs. Sheldon and Ellegren (1996) proposed that either females might be able to "identify" the sperm of a particular male and allocate it to a specific egg, or that the probability of each egg producing a particular sex may vary according to its position in the laying sequence. This could possibly be caused by variation in hormone levels during the laying period (Gil et al., 1999, but see Birkhead et al., 2000).

Several studies have identified a significant relationship between chick sex and laying order, offering support for the second hypothesis (Ankney, 1982; Dijkstra et al., 1990), although other similar studies have failed to find an effect (Cooke and Harmsen, 1983; Leblanc, 1987). The successful pursuit of extrapair copulations by either sex may vary in feasibility over time due to constraints such as mate guarding by either sex (Baltz and Clark, 1997) or local competition. The possibility of achieving a successful extrapair copulation at the right time may therefore be too unpredictable to allow the female to specify the sex of each egg independently. In addition, the low level of predictability concerning the outcome of fertilizations (Colegrave et al., 1995) may hamper the specificity of this mechanism.

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