Maternal Effects Contribute to the Superior Performance of Extra-Pair Offspring

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Summary

The explanation for extra-pair mating in female birds remains poorly understood and contentious [1–7]. Several leading hypotheses propose that females benefit indirectly by enhancing the genetic quality of their offspring, through good genes or genetic compatibility effects [1, 8, 9]. Supporting this idea, recent studies have identified a range of fitness-related traits for which extra-pair offspring (EPO) are superior to their within-pair (WP) half-siblings [10–21]. However, such performance differences may result from nongenetic maternal effects if EPO are positioned earlier in the laying order and benefit from the advantages of earlier hatching [22, 23]. Here we show that EPO are larger, heavier, and more likely to fledge than their WP half-siblings in a population of blue tits, Cyanistes caeruleus. However, extra-pair paternity declined markedly with laying order, resulting in EPO generally hatching earlier. After correcting for variation in hatch time, none of the observed disparities between EPO and their WP half-siblings remained significant. These findings indicate that phenotypic comparisons between maternal half-siblings must consider potential hatching-order effects and suggest that the evidence for genetic benefits from extra-pair copulation may be less compelling than currently accepted. Moreover, the overrepresentation of EPO early in the laying order may help explain female extra-pair mating.

Results and Discussion

In many socially monogamous birds, individuals commonly participate in extra-pair copulations (EPC) that can result in extra-pair offspring (EPO) [1]. Although males may clearly benefit by siring more offspring, explanations for female participation in EPC remain contentious and poorly understood [1–7]. Most attention has focused on the idea that females engage in EPC to improve the genetic quality of their offspring, by mating with extra-pair males that are either genetically more compatible (e.g., more dissimilar) or of higher quality (the good-genes hypothesis) than their social partner [1, 8]. This hypothesis predicts that EPO should be both genetically and phenotypically superior to their within-pair (WP) half-siblings [1, 9] and has been tested by comparing the viability of EPO and within-pair offspring (WPO) from the same nest because, all else being equal, they should only differ in relation to the genetic contribution of the father [10, 11].

In accord with this prediction, numerous previous studies have revealed that EPO are superior to their maternal half-siblings in a range of fitness-related traits including growth rate [12, 13], fledging body mass, size and/or condition [11, 14–16], survival to fledging [10, 17], immune function [12, 18, 19], and recruitment to the local breeding population in subsequent years [20, 21]. Collectively, these studies represent the strongest support for the idea that females engage in EPC to improve the genetic quality of their offspring [1, 6]. However, such performance differences could also arise through nongenetic effects if EPO tend to be placed earlier in the laying sequence [22, 23].

In this study, we examined the relationship between paternity and laying order in blue tits, Cyanistes caeruleus, by hatching out 154 wild-collected clutches in incubators (see Experimental Procedures). Individual chicks were returned to their nest soon after hatching, allowing us to assess whether position in the laying order favored the performance of EPO compared to their WP half-siblings. Blue tits are well suited to address this question because (1) they produce large clutches, with a mean of 11.5 ± 2.1 (SD) in this study (2); EPO are common in blue tits [10, 17, 27–29], typically occurring in over 40% of broods; and (3) clutches often hatch asynchronously over several days, resulting in distinct size hierarchies and frequent mortality of smaller, late-hatching chicks [30, 31]. Furthermore, previous studies in blue tits have reported that EPO are larger and heavier in some circumstances [16] and are more likely to fledge than their WP half-siblings [10, 17].

Laying Order and Paternity

Among the 122 clutches for which both social parents were caught, 12.8% of the 1176 genotyped offspring were sired by extra-pair males, and 46.7% of these clutches contained at least one EPO. Within the 55 mixed-paternity clutches, the proportion of EPO declined markedly with laying order ($\chi^2 = 49.04$, $p < 0.001$; Figure 1; see also Table S1 available online, models 1a and 1b). Overall, 74% of EPO were positioned within the first half of clutches. The strong relationship between paternity and laying order did not vary with year of the study; sex of embryo; or laying date, size, or hatching asynchrony of the clutch ($p > 0.30$ for the interaction between these variables and laying order; Table S1, models 1a and 1b).

Hatching Time and Paternity

The hatching asynchrony of clutches in incubators ranged from 9.5 to 127 hr (mean = 53.4; SD = 22.2; n = 118 clutches), which was similar to that observed among naturally hatching clutches in the same population in the 2008 breeding season.
Actual laying order was transformed to a scale of 0 to 1 ("relative laying order") to standardize for variation in clutch size (see Experimental Procedures). Points represent mean values of the raw data, separated into ten equally distributed categories of relative laying order, together with the binomial standard error. The solid line shows the model-predicted decline in probability of extra-pair offspring with relative laying order (see Table S1, model 1a for full model details). A similar relationship was evident with actual rather than relative laying order (see Table S1, model 1b).

Within clutches, the average chick hatched 15.87 hr (SE = 0.64; n = 118 clutches, 1217 hatchlings) after the first chick (or chicks). There was generally a strong, nonlinear increase in hatch time (hr since hatching chicks) after the first chick (or chicks). There was generally a strong, nonlinear increase in hatch time (hr since hatching). The gradient of this relationship was dependent on the level of hatching asynchrony ($\chi^2 = 50.22; p < 0.001$; Figure 2; Table S1, model 2a). Within mixed-paternity broods, EPO hatched an average of 9.28 ± 2.12 hr earlier than their WP half-siblings ($\chi^2 = 21.22; p < 0.001; n = 43$ broods, 419 hatchlings; Table S1, model 2b). Inclusion of hatching asynchrony in this model revealed that the difference in hatch time between EP and WP half-siblings increased with hatching asynchrony ($\chi^2 = 4.68; p = 0.03$; Figure 3; Table S1, model 2c). However, both the mean difference in hatch time and interaction with hatching asynchrony were absent after statistically controlling for the effect of laying order ($p = 0.48$ and 0.71, respectively; see Table S1, model 2d). Hence, there was no evidence for a difference in the rate of embryonic development between EPO and their WP half-siblings.

**Hatching Time, Paternity, and Nestling Performance**

An average of 69.5% ± 3.1% (SE) of hatchlings (n = 141 broods, 1309 hatchlings) survived to brood age 15 (oldest chick [or chicks] = day 15), several days prior to the usual age of fledging. The probability of survival to brood age 15 declined markedly with hatch time (Figure 4A; Table S1, model 3a). Within mixed-paternity broods, EPO had a greater likelihood of surviving to brood age 15 compared to their WP half-siblings ($p = 0.04; n = 49$ broods), but this difference was no longer significant ($p = 0.48$) after correction for variation in hatch time (Figure 5; Table S1, models 3b and 3c).

**Comparisons between Within-Pair and Extra-Pair Offspring**

In line with previous studies (see references above), including several on blue tits [10, 16, 17], we found that EPO were larger, heavier, and more likely to fledge than their WP half-siblings. However, EPO were three times more likely to be produced in the first half of the clutch, translating to EPO hatching earlier than their WP half-siblings among asynchronously hatching clutches. Moreover, this difference in hatch time completely accounted for the disparities we observed in survival and wing length, whereas the differences in tarsus length and body mass were still evident but were no longer statistically significant. These findings show that position in the laying order favored EPO in this population. Indeed, the real disparities in hatching time and survival were most likely greater than observed because some chicks disappeared from the nest before blood sampling, and these chicks were typically from eggs produced late in the clutch and presumably more likely to be WPO.

More generally, we suggest that most of the fitness-related traits for which EPO have previously been identified as superior to their WP half-siblings could be influenced by hatching time. Immune function is known to improve with nestling age, and earlier-hatching chicks exhibit a greater response to immune challenges than their younger siblings [32].

Within 15-day-old broods, body mass, wing length, and tarsus length all declined strongly with hatch time (Figures 4B–4D; Table S1, models 4a, 5a, and 6a). Consequently, within mixed-paternity broods, EPO had longer wings ($p = 0.04$) and tarsi ($p = 0.001$) and were heavier ($p = 0.01$) than their WP half-siblings (Figure 5; Table S1, models 4b, 5b, and 6b). Moreover, the disparity in wing length increased with the degree of hatching asynchrony ($p = 0.01$; Table S1, model 4c). Again, however, these differences were not significant after correction for hatch time (Figure 5; Table S1, models 4d, 5c, and 6c), although EPO still tended to be heavier ($p = 0.09$) and have longer tarsi ($p = 0.07$).
Moreover, many studies, including our own, have shown that late-hatching individuals are less likely to fledge and/or are smaller and lighter than their older siblings [30–32]. Significantly, greater size and mass at fledging have been associated with greater future survival probability and reproductive success in a range of birds [32].

In the absence of genetic differences, there are at least two reasons to expect phenotypic disparities between early- and late-hatching chicks when brood members are measured at a given point in time. First, earlier-hatching chicks should be developmentally more advanced simply because they are older. Second, developmental rates may be modified by sibling competition for food, such that the youngest, less competitive chicks may not reach the same size or weight even after accounting for age differences [32, 33]. One approach to dealing with unknown variation in hatching time has been to statistically correct for age-related morphological traits [e.g., 12, 13, 18, 19]. Such corrections are not perfect because of (1) the nonlinear growth of most morphological traits, (2) likely differences in growth trajectories related to hatching time, (3) masking of genuine differences in growth, and/or (4) catch-up growth over the nestling period that may occur at the expense of immune function or other components of fitness expressed later in life [34–37]. Nevertheless, in the absence of exact hatching times, corrections for traits early in the nestling period, such as body mass and wing length, should go some way to controlling for disparities in size caused by variation in hatch time.

We observed that the difference in hatching time and wing length between EPO and their WP half-siblings increased across broods with the degree of hatching asynchrony. These findings reveal how variation in hatching asynchrony could also contribute to explaining some of the reported contextual effects of EPO superiority that have previously been attributed to genotype-by-environment interactions [e.g., 21]. Indeed, any circumstances that promote hatching asynchrony (e.g., reduced food availability) should magnify differences between EPO and WPO where there is a relationship (in either direction)

Figure 3. Difference in Hatch Time between Extra-Pair Offspring and Their Within-Pair Half-Siblings in Relation to Degree of Hatching Asynchrony in Mixed-Paternity Clutches of Blue Tits
Points show the difference between the mean hatch time of extra-pair offspring (EPO) versus within-pair offspring (WPO) in each clutch. The solid line shows the model-predicted difference in hatch time between EPO and WPO (paternity × hatching asynchrony interaction; \( \chi^2 = 4.68; p = 0.03 \); see Table S1, model 2c for details).

Figure 4. Hatch Time of Chicks in Clutches of Blue Tits Relative to the First Egg in Relation to Measures of Performance at Brood Age 15 Days
(A) Probability of surviving to brood age 15 days.
(B) Body mass.
(C) Wing length.
(D) Tarsus length.
These morphological measurements have been centered about brood means to correct for between-brood variation. The solid lines show the within-brood model-predicted relationships (see Table S1 for full model details). The error bars represent the standard error of the model-predicted values for each 10 hr interval in hatch time. The horizontal dotted lines in (B)–(D) represent zero deviation from the brood mean values.
Laying Order and Extra-Pair Paternity

Why Are Extra-Pair Offspring Overrepresented Early in the Laying Sequence?

Despite the confounding effects of laying order, our findings do not preclude the possibility that EPO are genetically superior to WPO. Genetic benefits to EPO may be expressed later in life (e.g., attractiveness, fecundity, longevity) or only in certain contexts related to environmental circumstances [12, 21]. Moreover, these indirect genetic benefits may be small and difficult to detect [1, 38]. Indeed, we observed a tendency for EPO to be both heavier and larger than their WP half-siblings, even after accounting for variation in hatching time, which may reflect real differences in genetic quality. Consequently, the early placement of EPO in the laying order may represent an adaptive maternal strategy to provide them with the advantages of earlier hatching [22, 23], assuming that EPC has some cost. (Otherwise, it would pay females to produce EPO throughout the laying order.) Alternatively, the opportunity for females to solicit EPC may decline once laying commences. For example, male mate guarding may become more effective because pair males have better information about their partner’s fertility status, or females may be constrained by the need to acquire resources for egg production [22].

Early production of EPO in the laying sequence is also consistent with the fertility-insurance hypothesis [23]. EPC before laying commences increases the likelihood that even the earliest laid eggs will be fertilized in the event of pair-

male infertility, whereas EPC performed after the start of laying can only fertilize later eggs. Furthermore, because viable sperm can be stored for an extended period [39], EPC motivated by fertility insurance could cease well before clutch completion, resulting in the observed decline in EPO over the laying sequence when the social male is fertile.

Rapid social mate switching early in the female’s fertile period could also account for some cases of apparent EPO but is unlikely to provide a general explanation because in all cases in which the identity of a pair was established during nest building (n = 16), the pair remained together. Similarly, mate switching during the prelaying period was not observed in a Belgian population of blue tits [10].

Conclusions

Our findings indicate that potential effects of laying order must always be considered when comparisons are drawn between maternal half-siblings and add to growing doubts about the strength of the empirical evidence for the genetic-benefit hypotheses of extra-pair mating ([3-6, 29], but see [2, 7]. At least one other study has reported a decline in EPO with laying order [23], but further studies are clearly warranted to assess the generality of this pattern. Indeed, variation in the nature of the relationship between paternity and laying order, coupled with population differences in the degree of hatching asynchrony and brood reduction, may contribute to explaining the inconsistent findings reported across the many studies that have compared EPO with WPO [6]. We have suggested several possible explanations for the decline in EPO with laying order, but most are not mutually exclusive and may be difficult to disentangle. Nevertheless, an explanation is clearly worth seeking because it may well help reveal why female birds engage in EPC.

Experimental Procedures

Field Procedures

This study was conducted over two seasons (2006 and 2007) on a nest-box population of blue tits in De Vosbergen estate near Groningen in the Netherlands (53°08’N, 06°35’E). All active nests were visited daily during laying, and eggs were numbered in the order of laying. Clutches were collected 1–2 days before hatching was anticipated (11–12 days after incubation commenced) and replaced with plastic model eggs to prevent desertion. Eggs were placed into individual compartments within incubators (Therbo-S) maintained at between 36.0°C and 37.5°C and between 50% and 80% relative humidity. Chicks hatching during the day (0630–2100) were returned to their nest within 2 hr, and those hatching overnight were returned by 0800 the following morning. Prior to return, chicks were individually marked by clipping the tip of one or two toenails. The time of return to the nest was regarded as a chick’s hatch time because feeding could only commence after this point. Brood age was defined as the number of days after the hatching of the oldest chick. Of 1732 eggs placed in incubators, 93% hatched successfully, 4% were infertile or showed minimal embryonic development, and 3% contained embryos but failed to hatch. Dead embryos were collected for subsequent molecular analysis.

When broods were 3 to 6 days old, all surviving chicks were blood sampled (5–25 µl) and any dead chicks were collected. Overall, 88.3% of 1688 hatchlings were genotyped. Chicks were fitted with uniquely numbered leg rings when broods were between days 8–15. When broods were 15 days old, we measured body mass (to the nearest 0.1 g), tarsus length (to the nearest 0.1 mm), and length of the third (outermost) primary feather (to the nearest 0.5 mm; referred to as wing length) of the remaining chicks. Social parents were caught with nest-box traps for blood sampling when broods were 8–10 days old.

Molecular Parentage and Sexing

We isolated DNA from blood samples via a chelex extraction procedure [40] and from tissue via a salt extraction procedure [41]. Sex of offspring was
determined as described in [42]. Polymerase chain reaction (PCR) products were separated by electrophoresis on a 2% agarose gel or an AB3730 DNA analyzer. To exclude paternity, we genotyped for six microsatellite loci: Pca3, Pca7, Pca8, and Pca9 [43]; Pocc6 [44]; and Pdo5 [45]. PCR reactions were carried out in a 10 µl volume containing 20–
50 ng DNA, 0.2 mM each deoxyribonucleotide triphosphate (dNTP), 0.25 µM forward and reverse primer, 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl2, and 0.125 U Taq DNA polymerase (Roche Diagnostics GmbH). The PCR program was 94°C for 1 min, 40 cycles of 94°C for 30 s, 55°C for 45 s and 72°C for 45 s, followed by 72°C for 2 min. Ts was 55°C for Pca3, Pca8, Pocc6, and Pdo5; 60°C for Pca7; and 53°C for Pca9. Fluorescently labeled PCR products were separated on an AB3730 DNA analyzer. Subse-
quently, allele lengths were determined with GeneMapper 4.0 software.

Using CERVUS 3.0 [46], mean exclusion probability of the six markers was calculated to be 0.99935 for the first (female) parent and 0.99997 for the second (male) parent (given the genotype of the first parent). Paternity of the social male was excluded if there were at least two mismatches between the genotypes of the putative father and offspring and the social mother matched the offspring for at least five of the six loci. Single mismatches between parents and 1176 offspring occurred at nine maternal and six paternal loci, most apparently as a result of single-repeat slippage mutations.

Statistical Analyses
Most analyses were conducted via hierarchical mixed modeling to account for the nonindependence and hierarchical structure of the data. These models all included a random component with clutch (or brood) identity at level two and egg (or chick) identity at level one. Binomial response models were fitted to examine the relationship between laying order (or hatch time) and the binary response variables of paternity and survival to day 15, whereas effects of hatching time on the day 15 clutch measurements of body mass, tarsus length, and wing length were examined via normal response models. These morphometric measurements were converted to a relative value by subtraction from the brood mean (i.e., centering) because we were only interested in within-brood variation.

The explanatory variable of laying order was transformed to a scale of 0 to 1 (division of actual laying position by clutch size) to standardize varia-
tion in clutch size, which ranged from 5 to 18 (mean = 11.47 ± 2.06 [SD], n = 186). This relative scale should be more biologically meaningful because only the last few eggs usually hatch appreciably later than earlier eggs (see Figure 2), regardless of clutch size. Nevertheless, we repeated these analyses with actual rather than relative laying order and also report these results. Laying order was strongly related to hatching order (see Figure 2), but we expected that hatching time relative to other chicks in the brood would have a more important influence on chick performance than laying order as such. Therefore, for each clutch in the brood, we quantified “hatch time” as the number of hours after the hatching of the first chick (or chicks) in that brood and used this in all analyses of offspring performance. In the models where hatch time was the dependent variable, it was log-trans-
formed to account for the strongly right-skewed distribution of these data.

The other explanatory variable tested in the models that varied at the egg (or clutch) level was offspring sex. We also tested clutch-level (or brood-
level) variables, including hatching asynchrony (interval in hr between when the first and the last egg in the clutch hatched), year (2006 or 2007), clutch size, and relative laying date (clutch initiation date centered about the median clutch initiation date for that year), in order to assess these variables’ possible interaction with laying order and/or hatch time. Potential nonlinear effects of laying order and hatch time were explored by inclusion of their squared terms.

Examinations of potential differences in hatch time, survival, and morpho-
metrics between EPO and their WP half-siblings were restricted to the subset of mixed-paternity clutches (or broods). These analyses were conducted in three stages. First, we constructed models including only paternity, offspring sex, and the proportion of EPO in the clutch (or brood), allowing us to estimate mean differences between EPO and their WP half-
siblings. Second, we included hatching asynchrony to assess whether it influenced the difference between EPO and their WP half-siblings (i.e., inter-
action between paternity and hatching asynchrony). Third, we introduced laying order or hatch time into the models (including their squared and interaction terms) to assess how they affected the observed difference between EPO and WPO. Because of our primary interest in within-brood comparisons, we also included “mean hatch time” of chicks in the brood to account for variation in hatching asynchrony between broods. The signifi-
cance of all explanatory terms was determined via the Wald statistic, which approximates the χ² distribution. Full and final model summaries are presented in Table S1. Sample sizes varied between analyses depending on the parameters included in the final model. All hierarchical modeling procedures were performed in MLwiN 2.02 [47].

Supplemental Data
The Supplemental Data include one table and can be found with this article online at http://www.cell.com/current-biology/supplemental/S0960-9822(09)00975-0.

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