Extra-pair paternity in Blue Tits (*Cyanistes caeruleus*)
depends on the combination of social partners’ age

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There is growing evidence that engaging in extra-pair copulations may be a strategy by which females can modify their initial mate choice if they are constrained by primary choice of the social mate. Several factors such as genetic similarity and adult phenotypic traits can affect extra-pair paternity (EPP) patterns, but the relative importance of these factors may vary among species. Moreover, interactive effects of male and female characteristics have rarely been considered. Here, we aimed to study how multiple parameters characterizing a breeding pair (i.e. genetic similarity between mates, partners’ age and laying date) predict the occurrence of EPP at the brood level. The study uses 4 years of data from a wild population of Blue Tits *Cyanistes caeruleus*. Contrary to predictions of the inbreeding avoidance hypothesis, we did not find a positive relationship between the occurrence of EPP and the relatedness of social mates. We also found that the probability of EPP was unrelated to laying date. However, EPP was predicted by an interaction of social partners’ ages. Specifically, EPP was less likely when old females were paired with old males in comparison to old females paired to young males. Our study suggests that the occurrence of EPP may be the result of behavioural interactions in which both male and female age are important for determining the outcome. Our results confirm the importance of considering the interactions of male and female characteristics in studies investigating EPP patterns.

Keywords: extra-pair copulation, inbreeding avoidance, long-term study, mate choice, passerine, relatedness, sexual selection.

Extra-pair copulation (EPC) constitutes a relatively common mating strategy among birds. However, this strategy is still not fully understood (Brouwer & Griffith 2019). Many hypotheses have been proposed to explain the functions of EPC; the most current adaptive hypotheses for extra-pair paternity (EPP) in birds have emphasized the benefits of EPP (reviewed in Griffith et al. 2002, Arnqvist & Kirkpatrick 2005, Akçay & Roughgarden 2007, Brouwer & Griffith 2019). Although males may clearly benefit from this behaviour, as it enables them to sire more offspring, the benefits to females are less obvious. Both direct and indirect genetic benefits in terms of genetic profits to the offspring are usually invoked to explain this intriguing female behaviour (Jennions & Petrie 2000, Blomqvist et al. 2002). Alternatively, Forstmeier et al. (2014) suggested that EPC behaviour

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may be a by-product of selection on other characteristics of the mating systems (i.e. being expressed through genetic correlations with selected traits that are important for sexual selection).

The one empirical pattern that has emerged over the past decade is the frequent finding that genetic similarity between social mates is positively correlated with the presence of extra-pair offspring (EPO) (Arct et al. 2015, Brouwer & Griffith 2019). Hence, extra-pair mating may serve to increase offspring viability by allowing females to obtain sperm from less-related males already mated to another female. Nevertheless, recent meta-analyses have indicated that the effect of the degree of relatedness between mates on the incidence of EPP remained relatively weak (Arct et al. 2015), or was absent entirely (Hsu et al. 2015, Kiere et al. 2016). Brouwer and Griffith (2019) concluded that while inbreeding avoidance could be an explanation for EPP in some species, it might not be a general explanation for all species. A fruitful direction for future studies of EPP would be to specifically address the impact of inbreeding by sampling from populations with a well-known record of fitness costs resulting from mating with genetically similar partners (i.e. relatively more inbred populations).

Besides genetic similarity, other factors may also influence EPP. All meta-analyses to date clearly indicate that partners’ age may be another important factor modulating the occurrence of EPC behaviour (Akcay & Roughgarden 2007, Cleasby & Nakagawa 2012). Male age, in particular, appears to be an important determinant of EPP (e.g. Girndt et al. 2018). Indeed, recent meta-analyses have shown that extra-pair males are, on average, older than cuckolded males (Akcay & Roughgarden 2007, Cleasby & Nakagawa 2012) even though estimated effect sizes of such dependency exhibit marked heterogeneity (Hsu et al. 2015). What gives older males the competitive edge over younger males is unclear (Girndt et al. 2018). However, a common explanation is that male age reflects genetic quality via demonstrated viability, as predicted by the good genes hypothesis (Akcay & Roughgarden 2007). There is also growing evidence that female age affects the incidence of EPP in birds (e.g. Stutchbury et al. 1997, Ramos et al. 2014); in other words, older females are more likely to gain EPP than younger ones, which may be related to a female’s breeding experience, including mate sampling. Experience may also facilitate discrimination among potential mates (e.g. Doligez et al. 2005). On the other hand, older females may have more EPO because they are better at avoiding mate-guarding (Bouwman & Komdeur 2005). Clearly, more studies are needed if we are to understand the factors that shape both male and female age-dependent variation in EPP. Moreover, recent studies suggest that possible interactions of male and female age should be explicitly considered and accounted for in studies investigating EPP patterns (Lubjuhn et al. 2004, Bouwman & Komdeur 2005, Ramos et al. 2014, Michalková et al. 2019). Unravelling the identity of the breeding pair may shed light on the mechanisms influencing EPP.

To date, no study has considered all the breeding pair characteristics considered by recent meta-analyses in a single analysis. In particular, the age of social partners has rarely been considered in one analysis alongside other proxies of EPP occurrence such as genetic similarity, or life-history parameters characterizing the objective reproductive quality of a breeding pair (e.g. breeding success, date of breeding commencement). This makes generalizing the resulting outcomes difficult if these factors are not treated jointly in one model.

Here, we attempt to fill this gap by considering multiple parameters of breeding pairs simultaneously. We studied EPP in a nestbox population of Blue Tits Cyanistes caeruleus on Gotland, Sweden. Blue Tits are socially monogamous with biparental care and produce one clutch per pair per breeding season. However, EPP is relatively common in this species. In our population more than 40% of broods contained at least one EPO (Arct et al. 2013). Drawing on evidence from a previous study of this population, which showed that the genetic similarity of parents negatively affected offspring fitness-related traits (Arct et al. 2019), we tested whether genetic similarity between social mates is correlated with the presence of EPP. We expected a positive relationship between the genetic similarity of social mates and the incidence of EPP. Because laying date and an interactive effect of partners’ ages were previously shown to affect the incidence of EPC behaviour (Moller et al. 2003, Lubjuhn et al. 2004, Bouwman & Komdeur 2005, Václav & Hoi 2007, Ramos et al. 2014), these factors were also included in the final analysis. As male extra-pair activity may be constrained temporarily by mate guarding (Václav & Hoi 2007), we predict that EPP should be higher in late-
breeding pairs. Moreover, because both female and male breeding experience may affect the occurrence of EPP, for example older females may be more selective of social mates and older males may have a higher ability to guard (Whittingham & Dunn 2010, Girndt et al. 2018), we predicted lower rates of EPP in broods where both social parents were older.

**METHODS**

**Study species and study site**

Our study was conducted in an established nest-box population of Blue Tits in the southern part of Gotland (57°03’N, 18°17’E) in the breeding seasons of 2009–12. Blue Tits are small, territorial, passerine birds with maximum longevity recorded in the studied population of 7 years (Sudyka et al. 2016). From the end of April, we regularly inspected nestboxes to determine laying date, clutch size and hatching success. Laying date was expressed as days from 1 April (=1). Adults were caught inside nestboxes or using mist-nets while they were feeding 14-day-old nestlings. Birds were aged as yearlings (first-time breeders born in the previous year) or adults based on the presence of moult limits in the wing primary/greater coverts (Svensson 1992) and were sexed by the presence/absence of a brood-patch. Some birds could also be assigned to an age class using ringing data. Blood samples (c. 20 μl) were collected from all nestlings (2 days after hatching) and adults, and were stored in 96% ethanol for further genetic analyses.

**Genetic and paternity analyses**

DNA was extracted from blood samples using Chelex according to a standard protocol (Griffiths et al. 1998). To assess genetic similarity between partners we used 16 autosomal microsatellite markers (see Table S1). Individuals were genotyped by amplifying relevant loci in a polymerase chain reaction (PCR) using a Qiagen Multiplex PCR kit (Qiagen AG, Hombrechtikon, Switzerland) as described by Olano-Marin et al. (2010). We ran 1000 randomizations of the markers for the ‘heterozygosity by locus’ estimate of heterozygosity and estimated the average heterozygosity–heterozygosity correlation (HHC) coefficient and the 95% confidence intervals using the Rhh R package (Alho et al. 2010). To detect identity disequilibrium (correlations in heterozygosity among loci) due to variance in inbreeding, we also calculated the $g_2$ parameter, as this provides a more powerful statistic than HHC (Szulkin et al. 2010). Then, we assessed the expected power of our microsatellite markers set to detect general-effect heterozygosity fitness correlations using formulae from Miller et al. (2014; eqn. 5). There was no indication of inbreeding in our population: HHC and $g_2$ for markers were not significantly different from zero (i.e. their 95% quantiles overlapped zero) (all markers: $r_{HHC} = 0.009$, 95% CI −0.069 to 0.089; $g_2 = 0.0007$, 95% CI −0.001 to 0.003, $P = 0.23$). The expected power to detect heterozygosity–fitness correlations according to Miller et al. (2014) was small ($r^2 = 0.04$). We calculated relatedness between the breeding adults using the R-package DEMERELATE (Kraemer & Gerlach 2013) and used the Wang estimator (Wang 2002) as a measure of genetic similarity of the pair members. This estimator ranges from −1 to 1, with negative values indicating that individuals share fewer alleles than average (Thuman & Griffith 2005). The mean genetic similarity of our sampled adult population was −0.0096 (expected value is 0).

In EPP analyses, the number of microsatellite loci used to determine parentage varied between years. In 2009 and 2010, the paternity was identified using five microsatellite loci: Pca3, Pca4, Pca8, PmaTGAn45 and Titgata79 (Arct et al. 2013). Genetic parentage analysis in 2011 was determined by using 15 microsatellite loci (Arct et al. 2017). In 2012 we used five microsatellite loci (Pca4, Pca7, Pca8, PmaTGAn45, Titgata79). Nestlings were considered as EPO if their genotype mismatched that of their putative father’s at two or more loci.

**Statistical analysis**

We used a chi-square test to determine whether the occurrence of EPP differed between study years. The association between genetic similarity and the occurrence of EPP was tested using a generalized linear model with binomial error variance and a logit-link function, implemented in the R environment (R Core Team 2020). Brood type (i.e. with or without EPP, coded as 1 and 0, respectively) was a binary response variable. EPO constitute a small percentage of nestlings in the brood in our study population, and in most nests there was only a single such nestling. For this
reason, we did not use the proportion of EPO in the brood as the response variable. Moreover, taking into account the number of EPO in the response variable would have diluted the female-centric point of view we were taking: our question related to a binary decision of a female to mate or not outside the bonds of a social pair, not how many offspring are sired from such matings. Male and female age (first-year breeders versus older) and the year of study were included as categorical, fixed effects, whereas laying date and genetic similarity between social partners were entered as covariates (standardized with mean = 0 and standard deviation = 1). We tested only one interaction (i.e. female age × male age) due to explicit a priori predicted effects of the ages of social mates on the probability of EPP. When the interaction was significant, we performed post-hoc pairwise comparisons of group means for factors involved in the interaction using the `emmeans` package in R (Lenth 2020). We also fitted models including ‘brood size’ as a categorical variable but did not find evidence for an effect of ‘brood size’. We assume that having EPP is not a random process but comes from both female and male decisions, so this result is as expected. We therefore decided not to include this variable in the final analysis.

In our dataset we had multiple measures for seven males. These males appeared in multiple breeding seasons with different females. To account for repeated measures of males, we also performed a generalized linear mixed model analysis with male identity as a random effect (see Table S2). Inclusion of this random effect did not change the results (Table S2). There was missing information for some breeding pairs, which resulted in slightly varying sample sizes in different analyses.

**RESULTS**

Forty-five of 123 females (36.6%) produced at least one EPO. Eighty-nine of 1171 nestlings (7.09%) were sired by an extra-pair male. Among 45 females that produced EPO, 46.7% had only one EPO, 28.9% had two EPO, 17.8% had three EPO and 6.7% had more than four EPO.

There were no significant differences in rates of EPP between years ($\chi^2 = 0.789$, df = 3, $P = 0.85$, $n = 123$). We found that the probability of EPP in Blue Tit pairs was not affected by laying date or genetic similarity between pair members (Table 1). However, the probability of EPP among broods was explained by an interactive effect of female age and male age (Table 1). Specifically, this interaction meant that old females paired to old males had a lower level of EPP in comparison to young females paired to old males (post-hoc test: $z$-ratio = 2.37, $P = 0.018$; Fig. 1). Moreover, there was a weak tendency for old females to have higher levels of EPP when paired to young males than old ones (post-hoc test: $z$-ratio = 1.81, $P = 0.070$; Fig. 1). There were no differences in EPP when young males were paired with young and old females (post-hoc test: $z$-ratio = -0.15, $P = 0.88$; Fig. 1) and when young females were mated to young and old males (post-hoc test: $z$-ratio = -1.27, $P = 0.20$; Fig. 1).

**DISCUSSION**

We found that the probability of the occurrence of EPP in Blue Tit pairs was affected neither by genetic similarity of parents, nor by laying date. The only significant predictor of occurrence of EPP at the brood level was an interaction of social parents’ ages. In our study population, older females engaged less frequently in EPP when paired to older males in comparison with young females paired to old males. There was also a weak tendency for old females to be more unfaithful when mated to young males than old ones (Fig. 1). Placing the observed pattern in a wider context is not easy – evidence for associations

| Table 1. Predictors of extra-pair paternity in a Blue Tit population on Gotland from 2009 to 2012 ($n = 76$). Generalized linear model with extra-pair paternity (0/1) as the dependent variable, and laying date, parental genetic similarity, female and male age (first-year breeders versus older), and year of study as predictors. Reference levels absorbed in the intercept are: 2009 (year), young (male and female age). |
|-------------|------|--------|--------|--------|
|             | Estimate | se  | z value | $P$ value |
| Intercept   | -0.90 | 0.69 | -1.30  | 0.194   |
| Year of study (2010) | -0.28 | 1.05 | -0.27  | 0.786   |
| Year of study (2011) | 0.43  | 0.81 | 0.53   | 0.597   |
| Year of study (2012) | 0.49  | 0.76 | 0.65   | 0.517   |
| Male age    | 0.89  | 0.70 | 1.27   | 0.203   |
| Female age  | 0.10  | 0.68 | 0.15   | 0.877   |
| Genetic similarity | 0.40  | 0.26 | 1.50   | 0.133   |
| Laying date  | -0.41 | 0.35 | -1.17  | 0.242   |
| Male age * Female age | -3.04 | 1.38 | -2.200 | 0.028   |

Significant results are shown in bold type.
between EPP and the combinations of male and female ages in birds is scarce. In Pied Flycatchers *Ficedula hypoleuca*, Coal Tits *Periparus ater* and Common Reed Buntings *Emberiza schoeniclus*, EPP appeared most likely when old females paired with young males (Rätti *et al.* 2001, Lubjuhn, *et al.* 2004, Bouwman & Komdeur 2005, respectively); in the Blue-Footed Booby *Sula nebouxii*, EPP decreased with increasing age of the social partner in young females, whereas in old females (≥8 years) it increased (Ramos *et al.* 2014).

Clearly, existing evidence for such a relationship is mixed and equivocal. The optimal extra-pair mating strategy in relation to age may be different for each species, depending on its traits, environment and other constraints. Specifically, we may expect that an extra-pair mating strategy in relation to age may differ for long-lived and short-lived species. Hence, more longitudinal studies with detailed data on individual age (Michálková *et al.* 2019) are needed to understand the factors that shape age-dependent variation in EPP and to distinguish the evaluation of the effect of actual age and the potential effect of senescence (Michálková *et al.* 2019). This obviously presents a challenge for future studies exploring the occurrence of EPP.

The pattern observed in our study may be related to differences in both female mating preferences and male guarding. Females may universally prefer to mate with older males (Sundberg & Dixon 1996, reviewed by Griffith *et al.* 2002) because viability selection leads to older males being of higher genotypic quality than younger males (Andersson 1994). Furthermore, older males may be more experienced and consequently better able to coerce females into mating with them and/or may be better able to prevent their mates from engaging in EPC (Westneat & Stewart 2003). For example, Green *et al.* (2002) found that male age influences mate-guarding behaviour in Brown Thornbills *Acanthiza pusilla* in such a way that older males tended to respond more aggressively to intruders. On the other hand, the age of breeders and territory quality tend to be correlated, with older breeders occupying the highest quality territories (Ferrer & Penteriani 2003), which may subsequently drive patterns of EPP (e.g. Hoi-Leitner *et al.* 1999). Unfortunately, our study design does not allow us to distinguish between those two different strategies. Future research that examines which sex initiates and controls EPC may help to explain our results.

Our previous results may indicate that in this study population, avoidance of mating with genetically similar individuals should be favoured (Arct *et al.* 2017, 2019). Specifically, we showed that the genetic similarity of social parents negatively affected offspring performance (Arct *et al.* 2019) because genetic similarity of parents negatively affected offspring immune responses. Moreover, we found a positive relationship between individual heterozygosity and body mass of female nestlings 14 days after hatching (Arct *et al.* 2017). Both these phenotypic traits play an important role in determining individual survival prospects (e.g. Cichoń & Dubiec 2005). We expected a positive relationship between genetic similarity of social mates and incidence of EPP, but we failed to support the inbreeding avoidance hypothesis in the present study. Although insufficient sample size is always a possible explanation for non-significant findings, it is unlikely that type II error alone is the reason for our results because our samples were comparable or larger than those of similar studies with significant findings (reviewed by Arct *et al.* 2015). On the other hand, the selection pressure for females to be choosy with respect to relatedness may be low in our study population.
because the risk of inbreeding is low (Arct et al. 2017, 2019). However, even in a population with a low rate of inbreeding, we can expect that high genetic similarity of mates reduces offspring fitness (which we observed in our previous studies; Arct et al. 2017, 2019) probably because homozygosity leads to the expression of recessive deleterious alleles.

Several previous studies have documented the importance of timing of breeding on occurrence of EPP (Stutchbury & Morton 1995, Václav & Hoi 2007, Canal et al. 2012, Tomotani et al. 2017). In contrast, our results and those of other studies (e.g. Girndt et al. 2018) did not confirm that laying date can directly influence patterns of EPP. There is not much evidence that laying date might be a general explanation for interspecific variation in EPP among socially monogamous species.

Our study shows that the occurrence of EPP in Blue Tits is best explained by a male and female age interaction but is independent of the degree of relatedness between the pair members. The study reveals the limitations of generalizing the results of EPP studies when the phenotypes of males and females are considered separately. Properly controlling for the effect of mates’ ages may offer one possible approach for reconciling mixed results from different EPP studies. Ideally, further studies should incorporate multiple parameters of breeding pairs when looking for proximate causes of EPP in wild bird populations.

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**ETHICAL NOTE**

Adults were captured in a nestbox during the day while feeding nestlings. Blood sampling did not seem to have any adverse effects on the adult birds or on the chicks fledging. The captured birds were released within their territory close to their breeding box. The data upon which this study is based were obtained following the Swedish guidelines for work on natural populations and under a permit from the Swedish Ringing Centre at the Museum of Natural History in Stockholm (permit no. RC712 to S.M.D.).

**AUTHOR CONTRIBUTIONS**

Aneta Arct: Conceptualization (lead); data curation (equal); formal analysis (lead); funding acquisition (lead); writing – original draft; investigation (lead); methodology (lead), project administration (lead). Szymon M. Drobniak: data curation (equal); formal analysis (supporting); writing – review and editing. Samantha Mellinger: data curation (supporting); formal analysis (supporting). Rafał Martyka: formal analysis (supporting); methodology (supporting); visualization (lead); validation writing – review & editing. Lars Gustafsson: resources; supervision; writing – review and editing. Marisuz Cichoń: resources; writing – review and editing.

**Data Availability Statement**

Data analysed in this paper will be accessible through Dryad.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Microsatellite loci used in this study and their characteristics.

Table S2. Predictors of extra-pair paternity in a Blue Tit population of Gotland from 2009 to 2012.