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Superiority of extra-pair offspring: maternal but not genetic effects as revealed by a mixed cross-fostering design

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Abstract

Extra-pair copulations (EPC) are the rule rather than an exception in socially monogamous birds, but despite widespread occurrences, the benefits of female infidelity remain elusive. Most attention has been paid to the possibility that females gain genetic benefits from EPC, and fitness comparisons between maternal half-siblings are considered to be a defining test of this hypothesis. Recently, it was shown that these comparisons may be confounded by within-brood maternal effects where one such effect may be the distribution of half-siblings in the laying order. However, this possibility is difficult to study as it would be necessary to detect the egg from which each chick hatched. In this study, we used a new approach for egg-chick assignment and cross-fostered eggs on an individual basis among a set of nests of the collared flycatcher *Ficedula albicollis*. After hatching, chicks were ascribed to mothers and therefore to individual eggs by molecular genetic methods. Extra-pair young predominated early in the laying order. Under natural conditions, this should give them a competitive advantage over their half-siblings, mediated by hatching asynchrony. However, we experimentally synchronized hatching, and after this treatment, extra-pair young did not outperform within-pair young in any studied trait including survival up to recruitment and several indicators of reproductive success and attractiveness. We obtained only modest sample sizes for the last two traits and did not test for extra-pair success of male offspring. Thus, we cannot exclude the possibility of advantages of extra-pair young during the adult phase of life. However, our data tentatively suggest that the more likely reason for females' EPCs is the insurance against the infertility of a social mate.

Keywords: extra-pair paternity, fertility insurance, good genes, hatching asynchrony, maternal effects, offspring fitness

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Introduction

Females in most socially monogamous bird species also mate with extra-pair males (Griffith *et al.* 2002), but this is potentially costly for females. Promiscuity can expose females to sexually transmitted diseases (Sheldon 1993; Kempenaers & Schlicht 2010), and furthermore, their mates may reduce their parental care of broods containing extra-pair young (EPY, Arnqvist & Kirkpatrick 2005). Although males are not able to recognize their young (Kempenaers & Sheldon 1996), they are able to assess paternity loss owing to indirect cues such as female behaviour during the fertile period (Sheldon 2002). The wide occurrence of extra-pair mating suggests that these costs are outweighed by some benefits. By copulating with extra-pair males, females may benefit from increased access to food resources (Gray 1997; Tryjanowski & Hromada 2005), and broods containing extra-pair young may also be

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fed by extra-pair mates (Townsend *et al.* 2010). Females may also engage in extra-pair copulations (EPC) to insure against the infertility of a social mate (Sheldon 1994; Hasson & Stone 2009). Some of these direct benefits seem to be limited to special cases and life histories which is why most attention has been paid to potentially more universal indirect benefits. Females are hypothesized to improve the genetic quality of their offspring by extra-pair mating with a genetically more viable or compatible mate (reviews in Jennions & Petrie 2000; Neff & Pitcher 2005; Akçay & Roughgarden 2007; Kempenaers 2007; Mays *et al.* 2008). The strongest prediction of both of these hypotheses is the superior performance of EPY as compared to young sired by within-pair mates (WPY) (Sheldon *et al.* 1997; Griffith *et al.* 2002; Arnqvist & Kirkpatrick 2005; Hasson & Stone 2011).

The first studies comparing the quality of half-siblings appeared in the late 1990s (Kempenaers *et al.* 1997; Sheldon *et al.* 1997; Krokene *et al.* 1998) and since then many such tests have been published (reviews in Arnqvist & Kirkpatrick 2005; Akçay & Roughgarden 2007; Sardell *et al.* 2011). This research has shown that, on average, EPY do indeed outperform WPY, which suggests their genetic superiority but this indirect effect is very weak (Akçay & Roughgarden 2007). Such a small genetic benefit might be unable to outweigh the direct costs of EPC, which suggests that EPC are a male-driven strategy (Arnqvist & Kirkpatrick 2005). However, before ruling out the indirect benefits as a cause of EPC behaviour, it is vital to know whether the magnitude of the genetic effect has been estimated correctly. Three factors might have caused problems with the estimation of indirect benefits to date. First, genetic effects might be context dependent and thus manifested only under some environmental conditions (Sheldon 2000a; Schmoll *et al.* 2005; Garvin *et al.* 2006; O'Brien & Dawson 2007), hence the need for multiyear studies that test for genetic effects under a range of environmental conditions (Sheldon 2000a). Second, most previous studies were aimed at only one component of offspring fitness such as nestling quality or survival up to recruitment (Sardell *et al.* 2011), while the reproductive success of half-siblings was assessed in only one species (Schmoll *et al.* 2003, 2009). This large gap in our knowledge might contribute to an underestimation of the genetic benefits of EPC (Eliassen & Kokko 2008). Third, genetic effects might be confounded with maternal effects where, for example, EPY might be non-randomly distributed in the laying sequence (Krist *et al.* 2005). As many species initiate incubation before clutch completion (Clark & Wilson 1981; Stoleson & Beissinger 1995; Wang & Beissinger 2009), chicks from late eggs are delayed in development and thus disadvantaged in sibling competition (Magrath 1990; Stoleson & Beissinger 1995; Kim *et al.* 2010). If EPY were laid in early eggs, they would have a maternally derived competitive advantage compared to WPY, and this pattern was recently found in three passerine species (Johnson *et al.* 2009; Magrath *et al.* 2009; Ferree *et al.* 2010). So there is a need to control the laying/hatching order when comparing fitness-relevant traits of naturally occurring half-siblings (Kempenaers & Schlicht 2010).

An investigation into the distribution of EPY in the laying order requires an assignment of chicks to eggs. This is a major challenge for field studies as many chicks may hatch in a short time interval, but several methods can be employed to overcome this difficulty. Nests can be visited several times a day (Whittingham *et al.* 2007; Ferree *et al.* 2010) or eggs can be transferred to an incubator, while supplying nests with dummy eggs to prevent nest desertion, and then the hatched chicks returned to their nests (Krist *et al.* 2004; Magrath *et al.* 2009). Both of these methods are time-consuming. Chicks can also be assigned by putting the eggs into net sacks (Hořák & Albrecht 2007) or by injecting dye below the eggshell shortly before hatching (Sockman & Schwabl 2000; Rubolini *et al.* 2005), but these two methods are limited to larger species. Lastly, incubated eggs may be frozen, which can reveal the distribution of offspring sex (Cordero *et al.* 2000) or paternity (Cordero *et al.* 1999) in relation to laying order but is naturally not suitable if chick performance is of interest. Furthermore, in most species, this invasive method would raise serious ethical concerns.

The aim of this study was to test for the potential genetic benefits of EPC in the collared flycatcher (*Ficedula albicollis*) by comparing the performance of chicks sired by extra-pair mates and social mates. We conducted a mixed cross-fostering experiment to further reveal the distribution of EPY in the laying sequence. Eggs were cross-fostered among nests on an individual basis, so that each nest contained the same number of eggs as was in its original clutch, but each egg originated from a different donor nest. Consequently, this allowed us to assign chicks to mothers and therefore to individual eggs by molecular genetic methods. The potentially confounding impact of hatching asynchrony on chick quality was minimized as all eggs in mixed clutches started being incubated at the same time. The study spanned over four years that differed in environmental conditions which should add credibility to our test if genetic benefits are context dependent. Chicks were followed until recruitment, and some indicators of their attractiveness and reproductive success were tested for dependence on their paternity status.

Methods

Study population

This study was conducted in the Velký Kosíř area, Moravia, Czech Republic (49°32′N, 17°04′E, 300–400 m a.s.l.), in 2006–2010. Experiments were performed on a collared flycatcher population breeding in approximately 350 nest boxes in an oak (*Quercus petrea*) forest. The collared flycatcher is a small (13 g) hole-breeding passerine with sexually dimorphic plumage. Females are brownish with a small white wing patch, while males have more contrasting plumage with a white forehead patch and a larger wing patch. Females usually lay 4–8 eggs, and most of them start full incubation before clutch completion, usually on the penultimate or pre-penultimate eggs (Potti 1998). The early onset of incubation results in partial hatching asynchrony of the clutch (Krist *et al.* 2004; Rosivall *et al.* 2005).

Mixed cross-fostering experiment

The mixed cross-fostering experiment was performed in 2006–2009. Nest boxes with completed nests were visited daily, and each natural egg was exchanged for a dummy one on the day it was laid. It was recorded whether the eggs were warm on each visit. The first day that the eggs were warm was labelled as the start of the continuous incubation if the eggs were not found cold on later days. Natural eggs were measured by a digital caliper, and their volume was calculated according to Hoyt's (1979) formula to the nearest 1 mm^3. They were stored in a dry safe place located at one of the study plots. The day after the last egg in the focal nest was laid, the dummy eggs were replaced by the same number of natural eggs. Each of these natural eggs was originally laid in a different nest, excluding the focal one. As a result of this manipulation, each female started to incubate the whole experimental clutch of cross-fostered eggs at the same time. Experimental clutches were not created randomly from all available eggs; typically, eggs laid earlier were also used for earlier clutches to reduce the risk of their decreased viability while left unincubated (Cook *et al.* 2003; D'Alba *et al.* 2010; Wang *et al.* 2011). Eggs were stored and therefore left unincubated for 0–7 days, which is within the range of natural variation in this species. Storage time did not affect hatchability of eggs (mean storage time for hatched eggs: 3.82 days, $n = 937$, unhatched eggs: 3.59 days, $n = 27$, $F_{1,\,962} = 0.67$, $P = 0.412$).

The first nest in which the laying was completed was baited with the first eggs from other nests. If possible, eggs of the same laying order were used to create an experimental clutch. The use of earlier laid eggs was not an absolute rule as we aimed to increase the number of combinations of donor nests in experimental clutches (see Appendix S1, Supporting Information for an example of field protocol). This mixed cross-fostering design relies on the synchronized breeding of several pairs where the minimum requirement is to have eggs available from the number of nests equal to the clutch size of the female that just commenced laying. In our study population, it was possible to include nearly all nests in this type of experimental design.

Other field procedures

To detect the hatching day, experimental nests were checked from day 12 onward after experimental clutches were created. At 6 days of age, chicks were weighed (to the nearest 0.25 g), ringed and blood-sampled from the tarsal vein. By this method, only a small amount of blood was withdrawn (usually 1–5 μL) compared to the more common method of brachial venipuncture, which may have significant fitness costs (Brown & Brown 2009). The small amount of blood sampled was nevertheless fully sufficient for our aim, and we obtained genotypes from all blood samples. Unhatched eggs and dead nestlings were taken from nests and stored in ethanol. At 12 days of age, the width of the right wing web was measured twice by a thickness gauge (Mitutoyo Quick-Mini) and then injected with 0.1 mg of phytohaemagglutinin (PHA) in 20 μL of physiological saline solution. This procedure for the scoring of PHA immunity was applied only in 2006 and 2007. At 13 days, the chicks were reweighed, and the length of their tarsi (to the nearest 0.01 mm) and wings (to the nearest 1 mm) and the thickness of their wing webs (to the nearest 0.01 mm) were measured. The difference between the average thicknesses of the wing web before and after the injection was used as an estimate of the individual proinflammatory potential (see Vinkler *et al.* 2010). The nestling condition was estimated as residuals from the linear regression of the 13-day body mass on the tarsus length (body mass = −10.9 + 1.29 × tarsus length, $n = 711$, $R^2 = 0.34$).

During 2006–2010, collared flycatchers initiated (laid at least one egg) 704 breeding attempts on our study plots. We captured 520 male parents and 580 female parents while feeding nestlings. Ring identity of captured adults was used to identify which of the young from the experimental nests had been recruited to the study population. It is likely that some young that recruited to the breeding population were not recorded by us due to dispersal outside study plots and our inability to catch all breeding adults, such as due to early failure of the breeding attempt. However, we recorded 120 recruits from experimental nests giving

0.75 recruits per breeding attempt and local recruitment of 11.7% of laid eggs or 16.9% of fledged young. For comparison, in an extensively studied island population of this species, the average number of recruits per breeding attempt was 0.64 (Gustafsson 1989). High values of local recruitment in our population suggest that the status of most offspring was identified correctly and that the results on recruits are reliable.

Several traits were measured on recruits: (i) laying date (Julian day, 1st January = 1), (ii) clutch size, (iii) mean egg volume, (iv) body mass, (v) tarsus length, (vi) wing length, (vii) wing patch, calculated as the sum of visible white on primaries 3–8 as measured from the tips of the coverts to the distal part of wing (in mm), and (viii) forehead patch in males. Two pictures of the forehead patch were taken by digital camera. A ruler was aligned alongside the forehead patch on each picture, the white patch was manually encircled and the size of encircled area was computed by J-image software to the nearest 0.1 mm^2. All these procedures, including the photography, were performed twice, and the mean of the two measurements was taken as the size of the forehead patch. In experimental nests, both parents were blood-sampled by tibial venipuncture, and similar to the tarsal venipuncture in nestlings, only a small amount of blood (usually 1–5 μL) was withdrawn. All males in non-experimental nests were blood-sampled in 2007–2009.

Genotyping

DNA was extracted from blood and tissue samples using DNeasy® Blood & Tissue kit (Qiagen), and offspring and adults were genotyped at eight polymorphic microsatellite autosomal loci (Table 1). The microsatellite loci were amplified in a single multiplex PCR using fluorescently labelled primers and a Type-it® Microsatellite PCR kit (Qiagen). The reaction conditions used were 5 min. at 95 °C followed by 30 cycles of 30 s at 95 °C, 90 s at 65 °C and 30 s at 72 °C, with a final extension of 30 min at 60 °C. PCR products were mixed with GeneScan™–500 LIZ® Size Standard (Applied Biosystems) and analysed using ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems). Genotypes were then scored with the GeneMarker® version 1.9 software (Softgenetics), and loci characteristics based on allele frequencies were calculated using the Cervus version 3.0.3 (Field Genetics Ltd). The combined non-exclusion probability of this marker set was 5.67×10^{-4} for the first parent. However, locus Fhy310 had a considerable excess of homozygotes suggesting allelic dropouts or null alleles and was not used for inferences based on pairwise comparisons of offspring and candidate parents. The combined non-exclusion probability of the reduced set of seven loci was 1.48×10^{-3}.

Blood samples and genotypes were taken more than once from 147 birds owing to their breeding in multiple years or from repeated sampling of polygamous males in the same year. Some individuals were sampled twice (103), three times (37), four times (6) and one-five times, but only one genotype per individual was included for the above computation of allele frequencies and exclusion power. In total, 1235 individuals were genotyped, 1169 of them on all seven loci, 60 on six loci and six on five loci. Individuals that could not be genotyped at five loci were excluded from the analyses. The repeated genotyping of the same individuals was used to assess the frequency of genotyping errors. Two individual genotypes obtained in one sampling episode were barely readable and therefore were replaced with the genotypes obtained in the other sampling episode. Of the remaining 145 individuals, 127 (88%) had identical genotypes in all sampling episodes, 16 (11%) individuals differed at one locus and two (1.4%) differed at two loci. These errors can be ascribed to allelic dropouts or null alleles (11 cases) or a shift of 2–4 base pairs (nine cases).

Table 1 The characteristics of the marker set of eight microsatellite loci

Locus	*k*	*N*	Hobs	Hexp	*F*(Null)
Fhu2	20	1235	0.847	0.846	−0.0008
Cuμ04	20	1234	0.870	0.866	−0.0031
Fhy310	18	1227	0.726	0.883	+0.0968
Fhy405	28	1194	0.818	0.904	+0.0501
Fhy407	27	1234	0.919	0.910	−0.0052
Fhy428	24	1224	0.853	0.863	+0.0054
Fhy431	20	1235	0.873	0.866	−0.0040
Fhy452	32	1217	0.844	0.842	−0.0014

k, number of alleles; *N*, number of typed individuals; Hobs, observed heterozygosity; Hexp, expected heterozygosity; *F*(Null), estimated frequency of null alleles. Loci are described in Ellegren 1992 [Fhu2 (=PTC3)], Gibbs *et al.* (1999) (Cuμ04) and [Leder *et al.* (2008), Fhy310, Fhy405, Fhy407, Fhy428, Fhy431, Fhy452].

Parentage assignment

Owing to the nature of the mixed cross-fostering experiment, neither fathers nor mothers were known and maternity was assigned based on pairwise comparisons of offspring and candidate mother genotypes. The number of candidate mothers was equal to the clutch size in the focal nest, which ranged from four to eight. Conspecific brood parasitism does not occur in this population as confirmed by genetic data (Krist *et al.* 2005) and our observations that two eggs were not laid in any experimental nest during one day. Consequently, the set of candidate mothers was known with certainty, and for most nests, assignment of maternity was unequivocal by this pairwise comparison method. Typically, each offspring in the focal nest matched only one candidate mother on all loci, while all other potential combinations would involve a number of mismatches in two or more offspring–mother pairs (Appendix S2, Supporting Information).

For 17 of the offspring from eight nests, maternity was impossible to resolve with certainty as two or more offspring–mother pairs were equally likely (Appendix S2, Supporting Information). Another problem arose due to the lack of genotypes from some candidate mothers. If only one of the candidate mothers was ungenotyped for the focal nest, maternity could usually be resolved as all but one offspring matched the genotyped candidate mothers. Thus, the remaining offspring that did not match any genotyped candidate mother was assigned to an ungenotyped one. If two (n = 17) or three (n = 1) mothers were ungenotyped, the offspring that did not match any genotyped mother could not be simply assigned to the ungenotyped one.

For resolution of these cases, another approach was adopted where maternity could still be inferred based solely on an analysis of the genotypes of the candidate siblings. As the number of siblings ranged from four to eight, this analysis is more powerful than the pairwise mother–offspring comparison as it is based on a higher number of genotypes (see Walling *et al.* 2010). We ran the likelihood-based sibling analyses in the software Colony, version 2.0 (Wang 2004; Wang & Santure 2009), using also locus Fhy310. Information about the excess of homozygotes at this locus was included in likelihood calculations. For each offspring, Colony indicated the most probable parents labelled with arbitrary numbers as we did not include parental identities in these analyses. The results of these analyses confirmed the previous assignments made by offspring–mother pairwise comparisons in Cervus 3.0.3 (Kalinowski *et al.* 2007) and also resolved all controversies. For the 17 ambiguous offspring, the assignment to families was carried out based on the following results. (i) For nine offspring, Colony indicated that they were full-sibs with all other offspring of one candidate mother. (ii) Five offspring were indicated to be full-sibs of some of the offspring of one candidate mother and half-sibs with her remaining offspring, as expected for mixed-paternity broods. (iii) Three remaining offspring were indicated as half-sibs with only some offspring from one of the candidate mothers. The other offspring of this candidate mother were not indicated as sibs of the focal offspring. However, these seemingly unrelated offspring were half-sibs of those offspring related to the focal one. Of the 37 offspring with an ungenotyped mother, 20 passed into full-sib families, 12 into families with half-sibs and five into families which included some offspring unrelated to the focal one (according to likelihood algorithm).

Overall, for 846 offspring with genotyped mothers, 740 had no mismatch with their mother (87%), 99 had one mismatch (12%) and seven had two mismatches (1%). These figures are similar to those that compare genotypes obtained repeatedly for the same individuals indicating that most mismatches in mother–offspring pairs were probably caused by genotyping errors.

After maternity was determined for each offspring, paternity exclusion could begin where the distribution of the number of mismatches in offspring–social father pairs is given in Table 2. Males were considered genetic fathers if they had 0 or 1 mismatch with the offspring. For those with two mismatches, Cervus indicated a 95% probability for the male that fathered the offspring in three cases. These offspring were considered to be fathered by a social mate, and all other offspring were considered to be fathered by an extra-pair mate.

Statistical analyses

Only clutches with mixed paternity were used for the test of whether extra-pair young appear randomly in the laying order, and two analyses of this hypothesis were conducted. In the first analysis, the actual laying order and the relative size of the egg (egg volume minus mean egg volume of the clutch) were the fixed

Table 2 The distribution of the number of mismatches between 800 offspring and their social fathers

Mismatches	0	1	2	3	4	5	6	7
Cases	505	78	21	44	56	64	25	7

factors of interest, while the year and laying date in the nest of origin were included as fixed covariates. The identity of the original nest was the random factor, and the paternity was the response variable. As clutch sizes in mixed-paternity clutches ranged from four to eight (mean ± SD, 6.48 ± 0.678), the relative laying order might be a more relevant variable than the absolute laying order. Therefore, in the second model, actual laying order was replaced by the relative laying order (actual laying order divided by clutch size; Magrath *et al.* 2009; Ferree *et al.* 2010).

For the comparison of EPY and WPY performance, we used a suite of factors. These included the survival probabilities during three life stages, five morphological and one immunological trait of nestlings that predict post-fledging survival (Kruuk *et al.* 2001; Merilä *et al.* 2001; Moreno *et al.* 2005). We also compared the morphology of recruits to test whether the potential differences between within-pair and extra-pair nestlings persist until adulthood, although these adult traits are generally under weak selection (Przybylo *et al.* 2000). Furthermore, we tested the difference in three life history traits (egg size, clutch size and laying date) that affect reproductive success (e.g. Gustafsson & Sutherland 1988; Krist 2009). Specifically, laying date is under strong directional selection in this species (Sheldon *et al.* 2003), and egg size is sometimes used as a surrogate of female quality (Hõrak *et al.* 1997). Finally, we compared the size of two ornamental traits which plays a role in sexual selection (e.g. Sheldon & Ellegren 1999; Garamszegi *et al.* 2006).

Only the young originating from mixed-paternity clutches were used for the comparison of EPY and WPY performance. All statistical models where the response variable was nestling traits or offspring survival had a similar structure. Both the nest of origin and the nest of rearing were included as random factors, paternity as the fixed factor of interest, and the year and laying date in the nest of rearing as fixed covariates. Nests with complete failures were excluded when the response variable was egg hatchability and nestling survival. Models with a binary response variable had a logit link function, while those with a continuous response variable had an identity link function where the latter models were based on young that fledged. Models where the response variable was a trait measured on recruits had additional fixed covariates. All these models initially included the year when traits on recruits were measured, the age (in years) and the sex of the recruits (excluding single-sex models). The model on body mass of the recruits also included the age of the recruit's offspring at the time when the recruits were captured to control for mass recession during provisioning of nestlings (see Krist 2009). These models were simplified by backward elimination of non-significant covariates ($P > 0.1$). The natal year, the recruit age and the breeding year were intercorrelated; thus, the reduced models are not suitable for inferences about these three intercorrelated factors. They are, however, well suited for the inference about paternity effects, which was the main target of this study. Some recruits were captured in multiple years, but only data from one randomly selected year were included to avoid pseudoreplication. All above models were fitted in SAS 9.1, Proc Glimmix (SAS Institute 2003).

The relationship between the number of eggs laid after the start of continuous incubation and the proportion of EPY in mixed-paternity clutches was tested in Proc Genmod (SAS Institute 2003). The response variable was the proportion of extra-pair young in a nest. For each nest, the number of EPY was the event and the number of all offspring with determined paternity was the trial. The year and laying date in the original nest were added as covariates.

Presentation of results

The paternity effects on offspring performance are presented in the form of effect sizes. These effect sizes are the difference in survival probabilities between EPY and WPY and the standardized difference in mean value of traits measured on a continuous scale. The means and probabilities were the least square means adjusted for the covariates retained in the final models. The difference between means was standardized by the division of the standard deviation in the pooled groups. Confidence intervals for the difference in proportions were based on formulas given in Borenstein *et al.* (2009, p. 38) and those for the mean difference on formula 16 in Nakagawa & Cuthill (2007).

Results

Hatchability and hatching asynchrony

On an individual basis during 2006–2009, 1026 eggs coming from 160 nests (2006: 23 nests; 2007: 63; 2008: 44; 2009: 30) were cross-fostered between those nests. Four of these nests containing 23 eggs were abandoned during incubation, five eggs were accidentally broken and the hatching of another seven eggs was disallowed. Of the remaining 991 eggs, 937 hatched which gave us a hatchability rate of 94.6%. This figure is very close to hatchability in natural nests (94.0%) and nests in which whole clutches were cross-fostered (93.9%; Krist 2009) indicating that egg handling did not affect egg survival.

Most nests were visited daily around the time of presumed hatching. Between two subsequent visits, 78 of

146 (53.4%) nests completely hatched. This measure of hatching asynchrony is only a crude one and does not mean that nests which were only partially hatched at the time of our visit hatched asynchronously. Most of them probably hatched during a few hours, but we had accidentally checked them in the middle of this period. Based on this crude measure, hatching asynchrony was reduced in experimental nests compared to other nests in which eggs were left in nests during egg laying (57/280 = 20.4%). About 75% of females started continuous incubation of the dummy eggs before clutch completion (Fig. 1).

Distribution of extra-pair young

Genotypes at five or more loci were obtained for 941 offspring. Genotypes were lacking for 85 eggs for the following reasons: 39 chicks disappeared from the nest, 23 eggs did not show any sign of embryo development (labelled as infertile hereafter) and therefore were not genotyped (see Arnold *et al.* 2003), nine eggs disappeared from the nest, five eggs were accidentally broken by us, five samples were lost, and in four samples, DNA had degraded due to tissue decay.

The female parent was captured and genotyped in 143 of the experimental nests, while males were identified in 135. Furthermore, seven females and 209 males were captured and genotyped in non-experimental nests during the course of this experiment. In total, 1434 genotypes were obtained for 1235 individuals as some individuals were genotyped multiple times (see Methods).

The genotype of the social father was obtained for 800 of 941 genotyped offspring. Extra-pair fathers sired 214 of these offspring (26.8%). All young were sired by a social father in 58 nests, while in eight nests, all the young were sired by extra-pair males. Mixed paternity was identified in 69 nests (51.1%). In these nests, 253 young were sired by a social father and 168 by an extra-pair father (39.9%), and the paternity of 36 young was undetermined. Two social fathers were hybrids with the pied flycatcher, and in both of these cases, all the young were sired by extra-pair males.

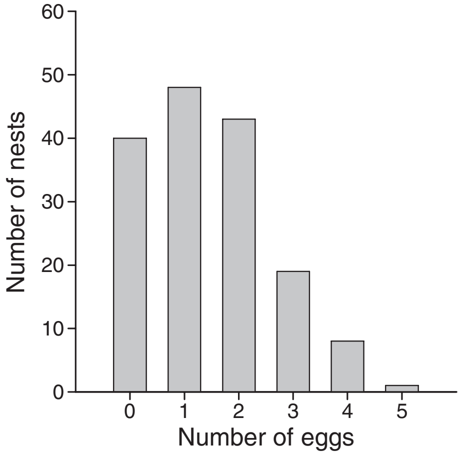

Fig. 1 The distribution of the number of eggs laid in experimental nests after the start of continuous incubation. Null means that continuous incubation started after the laying of the last egg.

Laying order, infertility and incubation

In mixed-paternity clutches, the proportion of extra-pair young decreased in laying order (Table 3). This pattern was similar for the actual and relative laying order (Fig. 2). EPY were found in eggs of the same size as WPY (Table 3).

In 16 nests, 23 infertile eggs were laid. The probability that a nest contained an infertile egg tended to be lower in nests where at least one young was sired by an extra-pair father (6/77 vs. 10/58, $\chi^2 = 2.83$, $P = 0.093$).

The proportion of EPY was not larger in nests where females started incubation later ($F_{1,63} = 0.40$, $P = 0.527$) and did not depend on the year ($F_{3,63} = 1.18$, $P = 0.318$) or the laying date ($F_{1,63} = 0.28$, $P = 0.600$).

Offspring performance

Five of the morphological and one of the immunological traits measured on nestlings were closely similar for EPY and WPY (Table 4, Fig. 3). These traits were measured on a large number of nestlings originating from mixed-paternity nests. Consequently, narrow confidence intervals excluded the possibility of large or even medium superiority of EPY at this stage. Using more long-term data, no significant effect of paternity was determined for any morphological, life history and secondary sexual ornamental traits measured on recruits (Table 5, Fig. 3). Sample sizes were smaller and therefore these effect sizes are less definite. However, point estimates were also generally small at this offspring stage (Fig. 3). Paternity was not a significant predictor of offspring survival during any of the three investigated life history stages (Table 4, Fig. 3). In sum, most effect sizes were close to zero. For a few traits, point estimates suggested medium or even large effects. However, these were invariable in a negative direction (i.e. WPY > EPY: forehead patch of males, recruitment probability). In contrast to the weak effect of paternity status, nestling morphology, immunity and survival varied between years and also depended on the time of the season (Table 4).

Table 3 The summary of models relating paternity to environmental and maternal variables in the nest of origin

		Absolute laying order					Relative laying order				
	NDF	DDF	Estimate	SE	*F*	*P*	DDF	Estimate	SE	*F*	*P*
Nest of origin			*0.277*	*0.178*				*0.278*	*0.178*		
Intercept			−3.86	5.34				−3.49	5.36		
Laying order	1	413	−0.227	0.0601	14.29	<0.001	413	−1.53	0.405	14.35	<0.001
Relative egg size	1	413	−0.000360	0.00166	0.05	0.828	413	−0.000350	0.00167	0.04	0.835
Laying date	1	65.5	0.0358	0.0433	0.68	0.412	65.6	0.0332	0.0434	0.58	0.448
Year	3	64.7			0.89	0.451	64.3			0.95	0.420

The random part of the models is in italics.

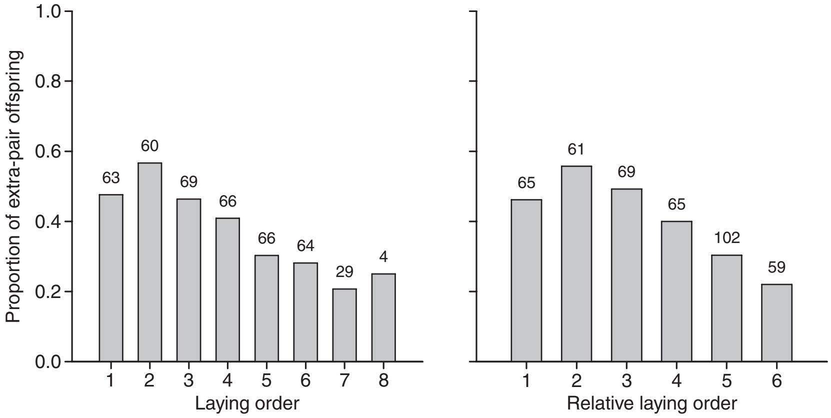

Fig. 2 The proportion of extra-pair young in mixed-paternity clutches in relation to laying order. Laying order is given as an absolute and relative value. Relative laying order is the actual laying order divided by the clutch size. The resulting continuous variable is separated into six categories to show the pattern in a clutch of modal size. The number of offspring in these categories was made approximately equal under the condition that the same value of relative laying order was not divided into two adjacent categories. The number of eggs with determined paternity is given above the bars.

Discussion

The proportion of EPY decreased with the laying order and 75% of the time females began to incubate before clutch completion, which usually led to hatching asynchrony of the brood. Therefore, under natural conditions, the occurrence of EPY in early eggs could give them a competitive advantage compared to later hatched WPY. Consequently, this maternal effect would confound an estimation of the genetic effect as might have been the case of our previous non-manipulative study (Krist *et al.* 2004). In that study, we were unable to control for hatching asynchrony when testing for the difference in survival probability between EPY and WPY. We found higher survival of EPY (Krist *et al.* 2004) that hatched from early eggs in the laying sequence (Krist *et al.* 2005), but in contrast to this result, we found no significant paternity effect on nestling morphology after hatching asynchrony was statistically controlled for (Krist *et al.* 2004).

In the present study, the confusing effect of hatching asynchrony was experimentally controlled for as females were forced to start incubation of all eggs at the same time. Extra-pair young did not outperform WPY in any studied trait in this large set of nests, suggesting that females do not obtain genetic benefits from extra-pair copulations. However, several issues need to be considered before ruling out this genetic-benefits explanation.

No genetic benefits from extra-pair paternity?

The usual approach to study genetic benefits of extra-pair paternity (EPP) is to compare performance of half-siblings that are raised in the same nest. However, in our study, half-siblings were raised in different nests, and we included nest of origin as a random factor in all analyses. Consequently, our analyses are similar in statistical principle to the usually employed paired tests (see West *et al.* 2007). However, as half-siblings were

Table 4 The dependence of offspring traits on paternity and two environmental factors

	Body mass at 6 days			Body mass at 13 days			Tarsus length			Condition			Wing length			PHA immunity		
	DDF	F/*estimate*	P/*SE*	DDF	F/*estimate*	P/*SE*	DDF	F/*estimate*	P/*SE*	DDF	F/*estimate*	P/*SE*	DDF	F/*estimate*	P/*SE*	DDF	F/*estimate*	P/*SE*
(A) Nestling morphology and immunity																		
Nest of origin		*0.219*	*0.0888*		*0.122*	*0.0654*		*0.0678*	*0.0224*		*0.0721*	*0.0448*		*0.0221*	*0.00625*		*0.00344*	*0.00242*
Nest of rearing		*1.14*	*0.225*		*0.910*	*0.176*		*0.149*	*0.0340*		*0.498*	*0.108*		*0.108*	*0.0167*		*0.00179*	*0.00217*
Residual		*0.543*	*0.0844*		*0.783*	*0.0912*		*0.150*	*0.0181*		*0.615*	*0.0708*		*0.0237*	*0.00307*		*0.0168*	*0.00319*
Paternity	152	0.03	0.856	250	<0.01	0.977	235	<0.01	0.977	265	<0.01	0.991	173	0.01	0.927	98	0.01	0.909
Laying date	102	2.12	0.148	121	2.84	0.095	129	8.55	0.004	114	0.02	0.884	138	16.82	<0.001	44	0.34	0.561
Year	95	23.87	<0.001	114	9.02	<0.001	108	6.75	<0.001	104	3.88	0.011	133	3.95	0.010	42	3.45	0.070

	Hatchability			Fledging success			Recruitment probability		
	DDF	F/*estimate*	P/*SE*	DDF	F/*estimate*	P/*SE*	DDF	F/*estimate*	P/*SE*
(B) Offspring survival									
Nest of origin		–	–		*0*	–		*0*	–
Nest of rearing		–	–		*1.08*	*0.608*		*0.434*	*0.370*
Paternity	403	0.30	0.583	343	0.02	0.892	315	3.68	0.056
Laying date	403	0.34	0.562	175	1.91	0.169	124	3.62	0.059
Year	403	0.95	0.389	128	3.41	0.020	209	4.81	0.003

The analyses were restricted to young originating from mixed-paternity broods. Random variables and their associated values are given in italics; fixed variables and their associated values are given in common type. Values associated with random effects are variance estimates and standard errors. Values associated with fixed factors are the results of type III *F*-tests: DDF, denominator degrees of freedom; *F*, *F*-value; *P*, *P*-value. Statistical direction of paternity effect is given in Fig. 3. Laying date means the date of the first egg in the nest of rearing. Year was fitted as a categorical variable.

raised in different nests, they also experienced different competitive environments. Thus, we introduced noise in our data that was caused by the variable quality of rearing environments that possibly decreased the power of our test. However, this noise is unlikely to mask genetic superiority of EPY for the following reasons. First, we gathered a large sample size that, on the contrary, increased the statistical power of our tests. Our design introduced most of the noise into the morphological traits of nestlings as indicated by the high proportion of variance (40–70%) explained by the random factor 'nest of rearing' (see Table 4). These values were considerably smaller for nestling immunity (8%) and traits of recruits (0–31%) with the highest value seen for nestling wing length (70%). This test was performed on 193 WPY and 128 EPY from 69 mixed-paternity broods. If our test of paternity effects utilized only the remaining 30% of variation in wing length, its power might be comparable to the conventional test that utilizes all the variation but with a sample size 30% of our values. This sample size would be 58 WPY and 38 EPY from 21 mixed-paternity broods which are still reasonable values and comparable to many previous studies. Second, the non-significance of our tests was not caused by wide confidence intervals that would overlap zero despite the point estimates being positive. In fact, the point estimates of effect sizes were actually close to zero or negative (Fig. 3). The confidence intervals were narrow at the nestling stage while wider for traits measured on recruits. Therefore, we can safely conclude that no genetic benefits are manifested during the nestling stage. Although there was also no indication of positive genetic effects later in life, as the respective point estimates were generally negative, this possibility cannot be completely ruled out as the power of these tests was relatively small.

Apart from the limitation of increased noise in data, our design has two advantages over former approaches. First, it enabled us to control for hatching asynchrony and egg size that might otherwise cause bias in tests of genetic benefits (Magrath *et al.* 2009; Ferree *et al.* 2010). Second, eggs originating from 69 mixed-paternity clutches were distributed to 156 nests. If genetic benefits were context-dependent, the higher number of raising contexts would enhance estimation of the average effect of paternal genes which matters most for selection on female behaviour. To conclude, if mixed cross-fostering is performed with a high sample size, it represents a vital alternative to conventional approaches for estimation of paternal genetic benefits.

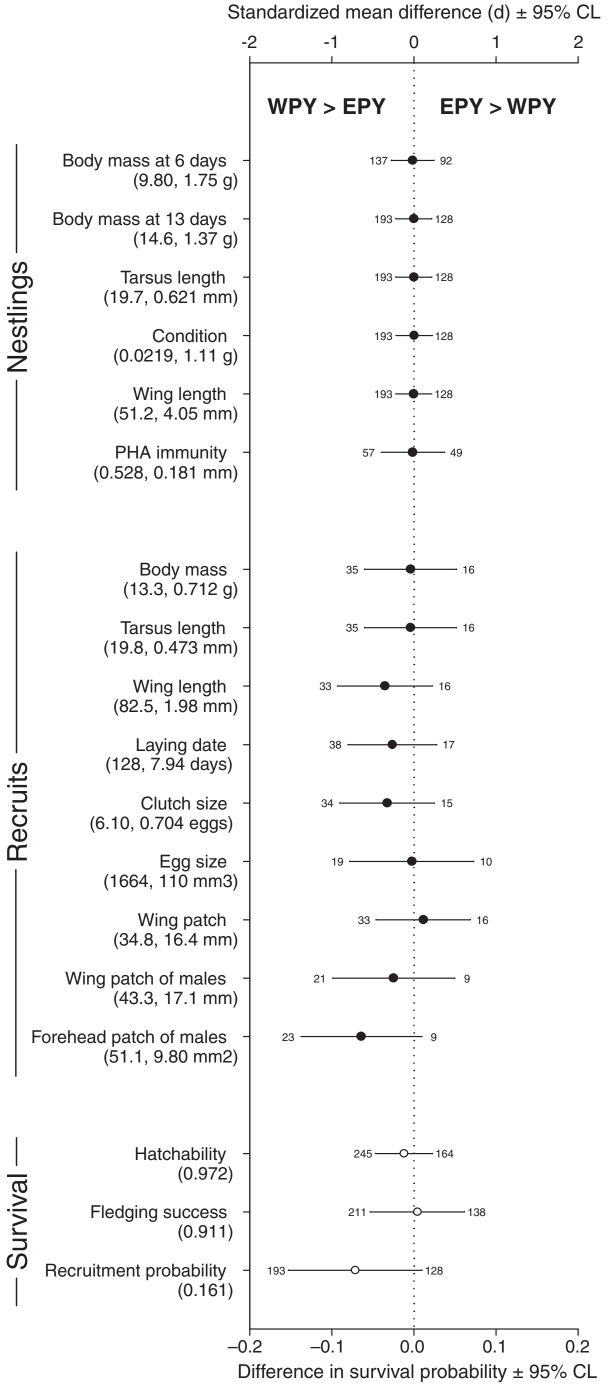

Fig. 3 The differences in performance of extra-pair and within-pair young originating from mixed-paternity nests. The number of WPY is given on the left and the number of EPY on the right of the bar. Numbers below trait labels are the mean and SD of the respective trait. Both statistics were computed on the pooled sample of all WPY and EPY from all nests. The presented SD was used for the standardization of the difference between means which is represented by filled symbols. Open symbols refer to the difference in survival probabilities.

As noted earlier, paternal genetic effects may be context-dependent. For example, they may only be manifested under poor environmental conditions (Neff &

Table 5 The dependence of recruit traits on paternity and a range of covariates

	Body mass			Tarsus length			Wing length			Laying date			Clutch size			Egg size		
	DDF	F/*estimate*	P/*SE*	DDF	F/*estimate*	P/*SE*	DDF	F/*estimate*	P/*SE*	DDF	F/*estimate*	P/*SE*	DDF	F/*estimate*	P/*SE*	DDF	F/*estimate*	P/*SE*
(A) Morphological and life history traits																		
Nest of origin		*0.244*	*0.189*		*0.125*	*0.0606*		*0.00247*	*0.00419*		*14.3*	*16.2*		*0.230*	*0.165*		*3503*	*4336*
Nest of rearing		*0.0230*	*0.0818*		*0.0742*	*0.0507*		*0.00203*	*0.00956*		*13.6*	*17.1*		*0.190*	*0.272*		*626*	*2752*
Residual		*0.216*	*0.144*		*0.0408*	*0.0309*		*0.0154*	*0.0102*		*43.5*	*21.8*		*0.242*	*0.302*		*5113*	*4703*
Paternity	**23**	**0.01**	**0.905**	**35**	**0.03**	**0.866**	**38**	**2.00**	**0.165**	**52**	**0.64**	**0.428**	**39**	**0.74**	**0.396**	**17**	**<0.01**	**0.948**
Laying date	28	0.45	0.506	**37**	**3.72**	**0.061**	20	2.28	0.147	30	0.01	0.929	34	1.30	0.263	8	<0.01	0.957
Year of hatching	37	0.50	0.609	**38**	**3.14**	**0.036**	**32**	**6.07**	**0.002**	39	0.10	0.909	33	0.08	0.927	21	2.48	0.109
Year of breeding	20	1.83	0.174	7	0.01	0.994	**29**	**10.70**	**<0.001**	44	2.17	0.106	31	1.03	0.392	12	1.57	0.248
Age	**24**	**4.69**	**0.010**	25	1.89	0.158	34	0.45	0.641	43	1.04	0.383	33	1.48	0.239	17	0.36	0.702
Sex	**26**	**7.72**	**0.010**	7	1.74	0.229	**37**	**2.96**	**0.094**	39	<0.01	0.996	45	1.27	0.266	20	2.15	0.158
Offspring age	38	1.60	0.213															

	Wing patch			Wing patch of males			Forehead patch of males		
	DDF	F/*estimate*	P/*SE*	DDF	F/*estimate*	P/*SE*	DDF	F/*estimate*	P/*SE*
(B) Ornamental traits									
Nest of origin		*21.8*	*34.7*		*0*	-		*59.7*	*23.7*
Nest of rearing		*19.5*	*41.4*		*0*	-		*14.0*	*10.5*
Residual		*64.1*	*46.7*		*62.8*	*17.8*		*0.445*	-
Paternity	**30**	**0.25**	**0.619**	**25**	**1.50**	**0.232**	**8**	**4.56**	**0.063**
Laying date	17	0.53	0.478	24	0.15	0.705	22	0.80	0.382
Year of hatching	**29**	**2.83**	**0.055**	19	2.32	0.107	20	0.74	0.491
Year of breeding	24	0.28	0.761	7	1.03	0.407	8	2.54	0.128
Age	**32**	**10.73**	**<0.001**	**25**	**34.12**	**<0.001**	**9**	**6.27**	**0.013**
Sex	**34**	**28.47**	**<0.001**						

The analyses were restricted to young originating from mixed-paternity broods. Random variables and their associated values are given in italics. Fixed variables and their associated values are given in common type if removed from the final model or in bold type if they remained in the final model. Values presented for removed covariates relate to the step just before their removal. Values associated with random effects are variance estimates and standard errors. Values associated with fixed factors are the results of type III *F*-tests: DDF, denominator degrees of freedom; *F*, *F*-value; *P*, *P*-value. Statistical direction of paternity effect is given in Fig. 3. Laying date means the date of the first egg in the nest of rearing. Years and age of recruits were fitted as categorical variables.

Pitcher 2005; Schmoll *et al.* 2005; Garvin *et al.* 2006). Consequently, studies limited to only good environmental conditions may not reveal any benefit of EPY over their half-siblings. This should not have affected our study as data were collected over 4 years that differed greatly in environmental conditions, as evidenced by the strong effect of year on offspring performance. Furthermore, offspring were raised under variable conditions within years as evidenced by the significant effect of laying date on their quality/survival. There were also four factors associated with our experimental design that might have changed the quality of the environment from the offspring's perspective. First, offspring were raised by foster parents, and there may be a coadaptation between offspring demands and parental supply (Wolf & Brodie 1998). If food provisioning is under parental control, as is likely in the collared flycatcher (Ottosson *et al.* 1997), cross-fostering could lead to a negative effect on offspring (Hinde *et al.* 2010). Second, broods were composed of non-related nest-mates which might induce higher sibling competition (Boncoraglio & Saino 2008). Third, eggs were returned to the nests a day after clutch completion which likely increased the energy spent for incubation and possibly reduced the females' capacity for chick provisioning. Finally, synchronous hatching reduced sibling hierarchy which might lead to increased sibling competition (Stoleson & Beissinger 1995; Kontiainen *et al.* 2010; but see Szöllősi *et al.* 2007). In sum, a mixed cross-fostering approach might have created a relatively competitive offspring environment. However, these subtle mechanisms, if indeed operating, should be of relatively minor significance compared to large annual and seasonal effects. Overall, maternal half-siblings were exposed to varying environmental conditions in this study, and context dependence therefore cannot account for the lack of difference between EPY and WPY.

Indirect genetic benefits should ideally be tested by a comparison of true fitness between maternal half-siblings. In a detailed long-term study of song sparrows (Sardell *et al.* 2011), female EPY were found to have lower recruitment and lifespan than WPY. Despite this, the greater fitness of EPY cannot be ruled out (Sardell *et al.* 2011) as survival may be traded for reproductive success (Hunt *et al.* 2004; Head *et al.* 2005). Therefore, reproductive success should be taken into account when looking for the genetic benefits of EPC (Eliassen & Kokko 2008). In this study, we focused on both survival and several indicators of reproductive success of WPY and EPY. Survival, life history traits (clutch size, egg size, breeding time) and secondary sexual traits (forehead and wing patches) were the same or smaller for EPY, which suggests that EPY do not outperform WPY in terms of either survival or reproductive success. Dependence of reproductive success on offspring paternity status was previously tested in a single species, the coal tit *Parus ater* (Schmoll *et al.* 2003, 2009), which found that EPY produced less offspring in their lifetime than WPY (Schmoll *et al.* 2009). One caveat of both the studies on coal tits and our study on flycatchers is a lack of data on extra-pair success of male offspring which is a component of reproductive success that has been shown to be a very important part of male fitness (Webster *et al.* 1995; Albrecht *et al.* 2007; Vedder *et al.* 2011). Without taking it into account, no definite conclusion about fitness benefits of EPC can be drawn (Schmoll *et al.* 2009). In this study, one indirect finding suggests that male EPY may not have increased access to extra-pair mates. We found that EPY offspring had the same size, if not smaller, secondary sexual ornament that is important for the female choice of both a social (Qvarnström *et al.* 2000; Hegyi *et al.* 2010) and an extra-pair mate (Sheldon & Ellegren 1999; Michl *et al.* 2002). As we did not find any indication for the genetic benefit of EPC, other explanations for involvement of females in this behaviour should be explored.

Other explanations for female infidelity

Females can sometimes obtain material benefits from extra-pair mates. For example, in great grey shrikes, *Lanius excubitor*, extra-pair males gave valuable courtship gifts to females before copulation (Tryjanowski & Hromada 2005). These gifts were mainly vertebrate prey items that represented up to 65% of the female daily energy expenditure. Female red-winged blackbirds, *Agelaius phoeniceus*, were allowed to forage on territories of neighbouring males with whom they had previously copulated (Gray 1997). In cooperatively breeding American crows, *Corvus brachyrhynchos*, extra-pair sires contribute to offspring provisioning which increased the offspring's survival rate (Townsend *et al.* 2010). Similar benefits are likely to be small or absent in the collared flycatcher. Males in this species do not defend food territories (Cramp & Perrins 1993) and although courtship feeding exists (Cramp & Perrins 1993), presented invertebrate prey probably does not cover a large proportion of the female daily energy expenditure. We also did not observe offspring provisioning by two males. Therefore, large material benefits from extra-pair copulations seem to be limited to special cases such as cooperative breeding (Townsend *et al.* 2010).

Females might also copulate with extra-pair mates to ensure against potential male infertility (Sheldon 1994; Hasson & Stone 2009). Recently, azoospermia, which is a complete lack of sperm in ejaculates, was found in 2–4% of males in two passerine species (Lifjeld *et al.* 2007). Other forms of functional male infertility such as oligospermia and asthenozoospermia were not investigated in that study. Similarly to azoospermia, these latter forms of male infertility may also contribute to female infidelity (Hasson & Stone 2009). Indirect evidence for male infertility and female insurance against it comes from studies that found an excess of broods with all offspring sired by extra-pair males (Krokene *et al.* 1998). This is also the case of our study where the proportion of these broods was relatively high (5.9%). Our observation that the number of EPY decreased in the laying order is consistent with the fertility insurance hypothesis. A single extra-pair copulation early in the female's fertile period could ensure the whole clutch against the potential infertility of a social mate. If a social mate was fertile, extra-pair young should disappear in later eggs as a result of subsequent inseminations from the social mate. We also found a tendency for less infertile eggs in broods with EPY which could tentatively suggest that EPC did indeed decrease the proportion of infertile eggs. However, there is a problem with this hypothesis as the opposite pattern (i.e. more infertile eggs in broods with EPY) also may be interpreted as support for fertility insurance (Wetton & Parkin 1991). One factor causing the conflicting predictions may be whether selection is currently operating or whether the population is already in equilibrium (Sheldon 1994). The second reason may be the ability of females to assess male infertility based on their appearance. If females were able to recognize the fertility of a social partner, only those with infertile partners could copulate with extra-pair males. In this case, a positive association between cuckoldry and infertility could arise. If females did not adjust their copulating behaviour to their partner's fertility, a negative association could arise. Recently, a positive correlation between sperm and plumage quality was found in the pied flycatcher, *Ficedula hypoleuca* (Calhim *et al.* 2009). This would suggest that females might also assess sperm quality in the collared flycatcher and therefore a positive correlation between infertility and EPY would be expected. Surely, more research is needed to elucidate whether there are fertility benefits of EPC in the collared flycatcher where special attention should be paid to sperm traits and their associations with the male phenotype. The data in hand suggest that fertility insurance might be more important for the evolution of EPC behaviour in the collared flycatcher than indirect genetic benefits.

Alternatively, females might not accrue any benefits from EPC and instead this behaviour might be driven by strong selection on male behaviour (Westneat & Stewart 2003; Arnqvist & Kirkpatrick 2005; Forstmeier *et al.* 2011). Males might either force or seduce (Holland & Rice 1998) females to behave in a maladaptive way. Behavioural observations on who initiates extra-pair copulations would be very useful in assessing this hypothesis (Kempenaers & Schlicht 2010). Unfortunately, it would be tremendously difficult to conduct such observations of the collared flycatcher in the field as both within-pair and extra-pair copulations are probably rare events (Michl *et al.* 2002). Moreover, female extra-pair behaviour might be genetically correlated with that of males, and females may not benefit from EPP even if they actively seek EPC (Forstmeier *et al.* 2011). This intriguing possibility remains to be tested in the wild.

Extra-pair young and maternal effects

In contrast to no evidence for paternal genetic effects, we have documented non-random distribution of EPY in laying order. Under natural conditions, hatching is partially asynchronous in the collared flycatcher (Krist *et al.* 2004; Rosivall *et al.* 2005) owing to incubation beginning before clutch completion, as was documented in this study. Consequently, under natural conditions, EPY would appear earlier in the hatching order than WPY. As the hatching order is a strong determinant of offspring performance (Krist *et al.* 2004; Rosivall *et al.* 2005), this would likely lead to non-genetic superiority of EPY. Recently, studies on three other passerine species found a predominance of EPY early in the laying or hatching order that resulted in their better growth (Johnson *et al.* 2009; Magrath *et al.* 2009; Ferree *et al.* 2010). After this maternal effect was statistically controlled for, differences between half-sibs disappeared (Magrath *et al.* 2009; Ferree *et al.* 2010). We controlled hatching asynchrony experimentally, and similar to those previous studies, we did not find any genetic effects on offspring quality. The quality of the young is considered to be the only ultimate test for genetic benefits of EPC (Hasson & Stone 2011; see also Puurtinen *et al.* 2009). Consequently, without the control for potential maternal effects, no strong conclusions about genetic benefits of EPC can be drawn (Kempenaers & Schlicht 2010). In the few studies that controlled for hatching asynchrony, genetic effects would be overestimated without this control. This was caused by the occurrence of EPY in early eggs. However, the distribution of EPY in laying order may differ both between and within species. For example, EPY occurred in early eggs from two independent samples obtained in our population in different years (Krist *et al.* 2005 and this study), while no such effect was found in Polish (Wilk *et al.* 2008) and Hungarian (Rosivall *et al.* 2009) populations of the species. If EPY occurred in late eggs (Riley *et al.* 1995), this could lead to an underestimation of paternal genetic effects owing to the counteractive effect of hatching asynchrony. Thus, the emerging view that the genetic benefits from EPC are small at best (Arnqvist & Kirkpatrick 2005; Akçay & Roughgarden 2007; Edler & Friedl 2008; Schmoll *et al.* 2009; Sardell *et al.* 2011) has no strong support as few previous studies have controlled for the maternal effects within broods.

The non-random distribution of EPY in mixed-paternity clutches probably arises as a result of the non-random distribution of copulations with social and extra-pair partners for which there are several potential explanations. First, it might be adaptive for females to put EPY in early or late eggs to facilitate or decrease the genetic-driven superiority of these offspring (i.e. differential allocation or compensation: Sheldon 2000b; Gowaty 2008; Horváthová *et al.* 2011). This explanation is unlikely for our system as EPY were not genetically superior. The second adaptive explanation may be that females copulate with an extra-pair partner before the onset of laying to ensure the whole clutch against the infertility of a social mate. Some pieces of evidence tentatively support this hypothesis. Other reasons for the non-random distribution of EPY in the laying order may be constraints imposed on female copulation behaviour. For example, if males guard their mates less in early fertile periods, females might seek extra-pair copulations mainly during this time. However, the opposite pattern of mate guarding is usually found (Westneat 1993; Pinxten & Eens 1997). Recently, another physiological mechanism that could potentially explain the excess of EPY in early eggs was proposed (Vedder *et al.* 2010). In that study, the addition of eggs into nests before the onset of laying induced a higher intensity of incubation and decreased the proportion of EPY in broods. This suggests that the female motivation to seek EPC decreases as the intensity of incubation increases, potentially leading to more EPY in early eggs compared to late ones (Vedder *et al.* 2010). Our correlative data did not support this prediction as the number of EPY was independent of female incubation during the egg laying stage. Thus, our data are consistent only with a scenario of the female actively seeking EPC for fertility insurance. However, more data on the behaviour of all three participants (female, social male and extra-pair male) are needed to elucidate the mechanisms leading to the excess of EPY in early eggs.

So far we have discussed only the non-random distribution of EPY in the laying order as a maternal effect that may confound the estimation of the genetic benefits from EPC. However, there are other maternal effects that might operate within broods. EPY might come from larger eggs, eggs with a higher concentration of hormones or carotenoids, or may be more provisioned by the parents. The last effect seems to be improbable as parents are apparently unable to recognize their own offspring (Westneat *et al.* 1995; Kempenaers & Sheldon 1996). Egg size (Slagsvold *et al.* 1984), yolk hormones (Gil 2008) and carotenoids (e.g. Saino *et al.* 2002; Cassey *et al.* 2005; Newbrey *et al.* 2008) often systematically vary with laying order. If the occurrence of EPY also systematically varies in the laying order, as shown in this study, it is likely that EPY may differ in egg size or composition from WPY. If such an association between prenatal nutrition and paternity was driven solely by laying order, it would be sufficient to control for this variable when comparing the performance of half-siblings. The finer targeting of nutrients based on paternity of ova seems to be unlikely (see Birkhead *et al.* 2000). Indeed, we found no association between egg size and paternity after the laying order had been controlled for. Although egg size (Krist 2011), yolk hormones (Gil 2008) and carotenoids (Saino *et al.* 2003; Biard *et al.* 2005; but see Remeš *et al.* 2007) affect offspring quality, their effect is likely to be small compared to the effect of the hatching order (Krist *et al.* 2004; Maddox & Weatherhead 2008; Mainwaring *et al.* 2010). To conclude, the hatching and/or laying order are the two maternal effects that most urgently need to be controlled for when assessing genetic benefits of EPCs via the comparison of half-siblings.

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M. K. is behavioural ecologist with interest in parental care and sexual selection in birds. P.M. is interested in population genetics of mammals and birds, and genetics of speciation.

Data accessibility

The data supporting all analyses and results of this paper have been deposited in Dryad repository under DOI: 10.5061/dryad.984b0528.

Supporting information

Appendix S1 An example of the field protocol of the mixed cross-fostering design.

Appendix S2 Maternity assignment file. For each genotyped offspring, all candidate mothers are listed together with the number of mismatches between maternal and offspring genotypes. Assignments that were possible only by sibling analyses in Colony software are marked in the file.