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DNA fingerprinting reveals a low incidence of extra-pair fertilizations in the lesser kestrel

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Abstract. DNA fingerprinting of socially monogamous, colonially breeding lesser kestrels, *Falco naumanni*, revealed that three of 87 (3.4%) nestlings were illegitimate, and all three came from a single nest of 26 sampled (3.8%). Extra-pair paternity may have arisen through either extra-pair copulation (EPC) or mate replacement. The maximum level of extra-pair fertilization (EPF) was low (3.4%) compared to that found in other socially monogamous species and was also lower than predicted according to the frequency of EPCs in a previous study (6.7%). Paired females depend strongly on male provisioning throughout the breeding season and may refuse to engage in EPCs to prevent withdrawal of parental investment by their mates. The DNA analysis also indicated that two nestlings in two different nests resulted from intraspecific brood parasitism. The frequency of parasitized broods revealed by DNA fingerprinting (7.4%, $N=27$) was higher than that inferred from egg-marking during nest inspections (3.8%, $N=52$). Pair copulation rates are high in the lesser kestrel but occur in a context of low sperm competition (i.e. low frequency of both EPCs and EPFs). Instead of assuring paternity, frequent copulations may function in females to assess their mate's condition. Alternatively, copulations may have a pair-bond function or may reduce the availability of an individual to other potential mates.

Until recent years, interpretation of animal behaviour was based on the assumption that the adults that rear the young are also the biological parents, particularly when studying reproductive success, mating systems and heritability of different traits (Simmons 1990; Avise 1994). This is not always the case, however. Birds, for example, used to be considered the paradigm of monogamy among vertebrates; more than 90% of avian species were considered monogamous (Lack 1968). Promiscuity in birds, however, is now believed to be the rule rather than the exception (Birkhead & Møller 1992).

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Individuals in supposedly monogamous species often adopt a mixed reproductive strategy (Trivers 1972). They are socially monogamous, but at least some seek copulations outside the pair bond. Both males and females may actively seek extra-pair copulations (EPCs), possibly resulting in a greater fitness for both sexes (for a review of benefits and drawbacks of engaging in EPCs, see Birkhead & Møller 1992).

Precise data on extra-pair paternity are needed to make evolutionary inferences about certain breeding strategies. Extra-pair copulation rates are not always good predictors of extra-pair paternity in birds, however (Dunn & Lifjeld 1994). In some species, EPCs are disproportionately more successful than pair copulations at fertilizing the eggs (e.g. indigo bunting, *Passerina cyanea*; Westneat 1987). Molecular techniques, such as allozyme electrophoresis or DNA fingerprinting, are needed to elucidate genetic parentage (Avise 1994).

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The lesser kestrel, *Falco naumanni*, is a small migratory falcon that breeds in the Palearctic, from the Iberian Peninsula to China, and winters in Africa (Cramp & Simmons 1980). In the Western Palearctic, lesser kestrels breed in urban colonies of up to 100 pairs, usually in buildings (Cramp & Simmons 1980). They are socially monogamous, although polygynous males have also been reported (Hiraldo et al. 1991).

The copulatory behaviour of lesser kestrels has already been studied in southern Spain (Negro et al. 1992a). Some males pursued a mixed reproductive strategy, with a 6.7% incidence of observed EPC attempts. The incidence of extra-pair fertilizations (EPFs) has not been determined, however.

The objectives of this study were (1) to estimate the incidence of extra-pair paternity in lesser kestrel broods by DNA fingerprinting, and (2) to examine whether the EPC rate previously reported by Negro et al. (1992a) was a reliable predictor of the level of extra-pair paternity in the species.

METHODS

Collection of Samples for DNA Analyses

The families were from 12 breeding colonies, ranging from two to 34 pairs, and located in Los Monegros (Aragón, northern Spain). This region is 700 km from the site where Negro et al. (1992a) conducted their behavioural study. We collected blood samples from 28 families in 1993. In 25 families we sampled the two presumptive parents. In the three remaining families, one parent (two males and one female) was not available for analysis.

We captured the presumptive parents at the nest when incubating or brooding small chicks to ensure that they were providing parental care and were not visitors unrelated to the nests. During their first week, we individually marked young at selected nests on the leg with a cloth strap, which we later replaced by a permanent metal band. The purpose of the early banding was to detect cases of nest switching by nestlings and their subsequent adoption, a phenomenon rather frequent in our population (J. L. Tella, M. G. Forero, J. A. Donázar, J. J. Negro & F. Hiraldo, unpublished data). We sampled young at 3–4 weeks of age. All adults and young were also colour-banded. We observed birds from a distance with telescopes to confirm that the previously banded adults were attending the nests and feeding their presumptive offspring.

We took approximately 0.4 ml of blood from the brachial vein using 1-cc syringes and 30-gauge needles. The blood was preserved in lysis buffer consisting of 0.01 M NaCl, 0.01 M EDTA, and 1% n-lauroylsarcosine, pH 7.5 (Seutin et al. 1991). Samples were stored at 4°C until processing.

DNA Extractions

Aliquots of the samples (0.25 ml) were mixed with 5 ml of 1 × SSC and centrifuged at 7000 rpm for 15 min. The resulting pellet was resuspended in 2 ml of 0.2 M sodium acetate (pH 7) and 100 µl of 20% SDS. After vortexing vigorously, we extracted the samples with 2 ml of a mixture consisting of equal parts of equilibrated phenol and chloroform:isoamyl alcohol (24:1). The samples were centrifuged at 2000 rpm for 20 min. The supernatant was transferred to a fresh tube and the phenol/chloroform extraction repeated. The resulting aqueous upper phase was mixed with two volumes of cold ethanol (100%). The precipitated DNA was recovered with a pasteur pipette, air-dried and dissolved in 0.5 ml of 5 mM Tris HCl, 0.1 mM EDTA (pH 7.4).

DNA Fingerprinting

Aliquots of 5 µg of DNA were digested overnight at 37°C with 25 units of the restriction enzyme HinfI and subjected to electrophoresis on 20 cm long 0.7% agarose gels at 29 V for 36 h.

The agarose gels were dried in a vacuum gel dryer for 1 h at room temperature and 30 min at 60°C. The dried gels were soaked in 0.5 M NaOH, 0.15 M NaCl to denature the DNA, neutralized with 0.5 M Tris HCl pH 8, 0.15 M NaCl and equilibrated with 6 × SSC.

The oligonucleotide probe $(GGAT)_4$ was labelled with [γ^{32}P]ATP using T4 polynucleotide kinase. The probe $(GGAT)_4$ was selected because it had been used successfully in a previous study on paternity of raptors (Wolfes et al. 1991). The hybridization mixture consisted of 5 × SSPE, 5 × Denhardt's solution, 10 µg/ml of herring sperm DNA and 1–2 × 10^6 cpm/ml of the ^{32}P-labelled oligonucleotide. Hybridization was carried out directly on the dried gels using 20 ml

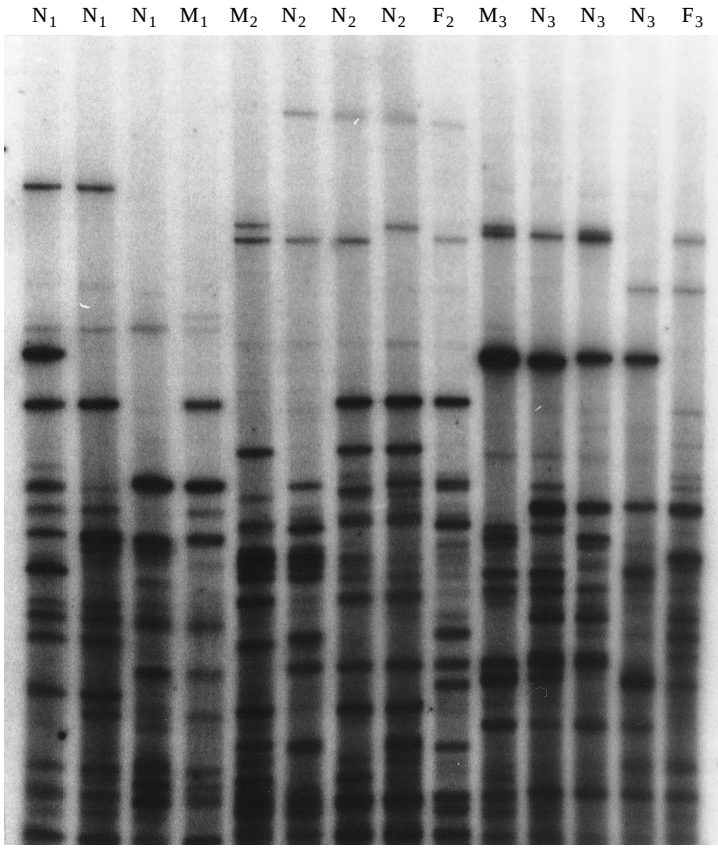

Figure 1. DNA fingerprints of two complete lesser kestrel families with no extra-pair paternity (2 and 3) and one single-parent family (1). Each vertical lane corresponds to one individual. F = female, M = male, N = nestling.

of the hybridization solution overnight at 42°C in a hybridization machine. Although restricted to oligonucleotides, the procedure is faster and more sensitive than the conventional Southern hybridization. The gels were washed twice for 30 min with 6 × SSC at room temperature. Autoradiography was conducted at room temperature with an exposure time of 3–4 days.

Assignment of Parentage

Comparisons of banding patterns were confined to lanes on the same gel, which held a maximum of 15 samples (Fig. 1). DNA from nestlings was flanked by that of their putative parents. Where both putative parents were available, parentage was determined by band exclusion analysis (Wetton et al. 1992; Decker et al. 1993; Sheldon & Burke 1994). In this analysis, bands in the offspring's fingerprint are matched to bands present in the parents. The presence of unattributable bands is the primary basis for determining mismatched parentage.

Where one of the parents was not available, we calculated band-sharing coefficients (BSC). These coefficients are calculated as $2(N_{AB})/(N_A + N_B)$, where N_{AB} is the number of bands shared by birds A and B, and N_A and N_B are the number of bands in birds A and B, respectively (Wetton et al. 1987). We calculated the distribution of band-sharing coefficient values for both unrelated individuals and first-order relatives according to the exclusion analysis (Fig. 2). We estimated the average level of band sharing for unrelated individuals in the population by comparing the putative parents and other adults run in adjacent lanes. Because some overlap existed between the two distributions, we used as the threshold limit for parentage exclusion

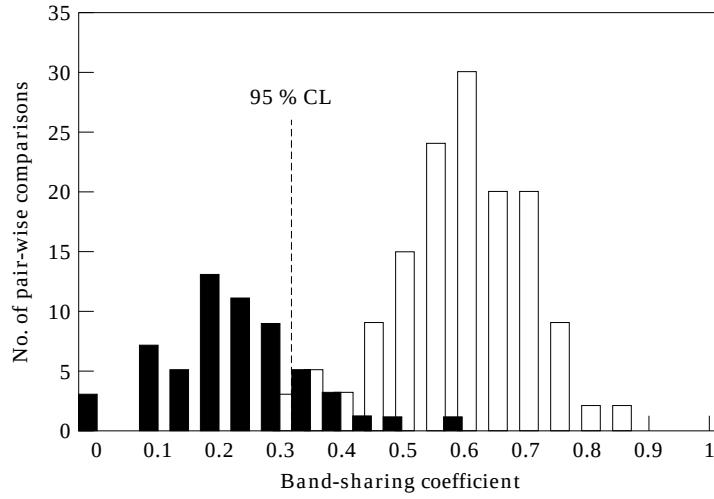

Figure 2. Frequency distributions of band-sharing coefficients for first-order relatives according to the band exclusion analysis, and between presumably unrelated individuals. The vertical discontinuous line marks the 95% lower confidence limit (CL) of the distribution for first-order relatives (threshold for paternity assignment).

the lower 95% confidence limit (BSC = 0.324) of the distribution for first-order relatives (Decker et al. 1993; Sheldon & Burke 1994).

We calculated the expected band-sharing coefficient (S) for first-order genetic relatives in our population according to the equation (Lynch 1991):

$$S = \theta + r\,(1 - \theta),$$

where θ is the background band-sharing coefficient, and r is the proportion of genes identical by descent between two individuals ($r = 0.5$ for parent–offspring or between full siblings).

RESULTS

Characteristics of the Band Patterns

A mean ± SD of 10.9 ± 2.6 scorable bands was analysed in the fingerprints of 147 individual lesser kestrels belonging to 28 presumptive families (Fig. 2). The mean band-sharing coefficient value for unrelated adults was 0.216 (Table I). Consequently, any two unrelated individuals had a probability of $0.216^{10.9}$, or less than 10^{-8}, of having identical fingerprints. The expected band-sharing coefficient between first-order relatives given a 'background' band-sharing coefficient of 0.216 is 0.608 (calculated according to Lynch 1991). This value was very close to the actual mean band-sharing coefficient values that we estimated for different categories of first-order relatives (Table I), and almost identical to the median of the distribution for first-order relatives (0.600, $N = 251$ BSC values).

A full linkage analysis was not carried out because large families of known parentage were not available (Amos et al. 1992). The distributions of band-sharing coefficients for father–offspring and mother–offspring did not differ significantly, however (Table I), suggesting that there was no predominant sex linkage.

Assignment of Parentage

According to the exclusion analyses, in 22 (88%) of 25 complete families, the attending parents were the true parents (Table II), including three families in which one nestling showed a single band that was not present in either of the parents. We believe that these unique bands were the result of mutation (Wetton et al. 1992; Lifjeld et al. 1993) because the band-sharing coefficients of the problem nestlings with their presumptive parents and siblings were all above our threshold limit for paternity exclusion (0.324). Thus, the

Table I. Band-sharing coefficients (BSC) between pairs of lesser kestrels whose relationship had been determined by band exclusion

Relationship*	Mean BSC	SD	*N*	Exclusion threshold†
Expected first-order relatives‡	0.608			
Observed first-order relatives	0.582	0.129	251	0.324
Male–offspring (a)	0.556	0.110	70	
Female–offspring (a)	0.587	0.101	80	
Full siblings (b)	0.596	0.157	101	
Excluded parent-nestling (c)	0.262	0.088	7	
Unrelated adults (c)	0.216	0.117	59	

*Relationships with a common letter (in parentheses) had BSC distributions that did not significantly differ ($P>0.05$; Kolmogorov–Smirnov two-samples test).
†95% lower confidence limit of the distribution for first-order relatives.
‡Calculated according to Lynch (1991).

Table II. Distribution of parentage in lesser kestrel broods

Parentage success	Incidence (no. nests)	Relative (no. extra-pair young/brood size)
No extra-pair fertilization	22	*
Extra-pair paternity	1	3/3
Extra-pair paternity and maternity	2	1/2, 1/4

Only nests where both attending parents were analysed are listed.
*Mean ± SD brood size was 3.04 ± 1.17.

mutation rate in the lesser kestrel might be approximately 4.1×10^{-3} per meiotic event, which is in the range reported previously for micro- and minisatellites (Burke & Brudford 1987; Burke et al. 1989; Westneat 1990; Lifjeld et al. 1993; Charlesworth et al. 1994).

We sampled only one of the two attending adults in three nests and concluded that they were true parents. In one case the available parent was male, and the band-sharing coefficients with his two putative offspring were 0.57 and 0.68, respectively. In the two remaining cases, the available parents were the females. The band-sharing coefficients with their putative offspring were 0.66, 0.63, 0.60 and 0.47 for one of the females, and 0.43 and 0.47 for the other female.

In one of the 26 families (3.8%) where the adult males were sampled, the presumptive father was unrelated to all three nestlings he was caring for. Thus, the number of extra-pair nestlings was three of 87 (3.4%). The three nestlings of the extra-pair brood showed four unmatched bands each. Although band-sharing coefficients with the mother were 0.7, 0.66 and 0.57, respectively, those with the presumptive father were 0.25, 0.14 and 0.21, well below our threshold for exclusion. We suspect, however, that this was a case of rapid female replacement in which the second female had been fertilized by an unknown male. In fact, 13 days before the female that we sampled started laying, we caught another female in the nest. This female was apparently close to egg laying because she had a swollen abdomen and weighed 171 g, a mass reached only by females during the mate-feeding period that precedes egg laying (Donázar et al. 1992).

In two families, one nestling in each was unrelated to both presumptive parents and to their siblings (two and three chicks, respectively), which in turn appeared to be true offspring. These two cases were probably the result of intraspecific brood parasitism (see Table II), and occurred in two of 27 nests (7.4%) in which the female was

sampled. Brood parasitism affected two of 91 (2.2%) of nestlings.

DISCUSSION

Some ecological and behavioural characteristics of lesser kestrels suggest that EPF could occur at a high rate. They breed colonially and thus availability of potential mates is high. In addition, males leave the females unattended at the colony precisely when the females are fertile (raptors, in general, are considered non-guarding species; Birkhead & Møller 1992). Furthermore, lesser kestrels show extremely high within-pair copulation rates (Negro et al. 1992a), which could be construed as a strategy by the males to counteract competitors' sperm (Birkhead et al. 1987). Extra-pair copulations have been observed, although at a relatively low rate (Negro et al. 1992a).

If frequent copulations were a paternity assurance tactic by the males, a positive relationship between paternity and copulation rate should be expected (Lifjeld et al. 1994). Our study, however, shows a low incidence of extra-pair paternity in a frequently copulating species. The same results have been obtained for merlins, *Falco columbarius* (Warkentin et al. 1994), and American kestrels, *F. sparverius* (M. Villarroel, D. Bird & U. Kuhnlein, unpublished data). In addition, copulations in raptors and other birds (e.g. Tortosa & Redondo 1992) extend over prolonged periods and probably occur outside the female's fertile period (Birkhead & Møller 1992; Negro et al. 1992a). An explanation for frequent copulation rates alternative to paternity assurance is thus warranted in those species (Tortosa & Redondo 1992; Lijfeld et al. 1994).

Assuming that all copulations, whether intra- or extra-pair, have the same probability to result in fertilizations, and that EPCs are evenly distributed in the population, the probability of EPFs (P_{EPF}) can be estimated from a binomial distribution as follows:

$$P_{\mathrm{EPF}} = 1\ (1-p)^n$$

where n is the size of the brood and p the frequency of EPCs. With $p = 0.067$ (Negro et al. 1992a) and an average brood size of 3.04 for the families that we studied, the expected number of nests with EPF is 4.94 of 26, compared to the observed value of 1 of 26. This overestimate may even be higher than apparent because in the single instance where EPF was observed, all offspring were from an extra-pair father and thus may represent replacement of the female by another one that had been fertilized elsewhere. Similarly, Pinxten et al. (1993) reported that three of six cases of extra-pair paternity in the European starling, *Sturnus vulgaris*, seemed to be due to rapid mate replacement occurring 1–2 days before egg laying.

We cannot exclude the possibility that the difference between expected and observed values occurred because we conducted the paternity and behaviour studies in different populations and different years. Wide annual and inter-populational variations have been observed in both the rates of EPCs and EPFs in the pied flycatcher, *Ficedula hypoleuca* (Gelter & Tegelstrom 1992).

Colonial species might suffer an increased incidence of extra-pair paternity due to enhanced opportunities for extra-pair encounters (Wittenberger & Hunt 1985; Birkhead et al. 1987; Simmons 1990; but see Dunn et al. 1994). In the lesser kestrel, however, the low level of extra-pair paternity can hardly be interpreted as a cost of coloniality. The only case of extra-pair paternity occurred in one of the smallest colonies, with just three pairs. Although some colonial species (i.e. tree swallows, *Tachycineta bicolor*; Lifjeld et al. 1993; shag, *Phalacrocorax aristotelis*; Graves et al. 1992) show a high incidence of extra-pair paternity, in others EPFs (European bee-eater, *Merops apiaster*; Jones et al. 1991; fulmar, *Fulmarus glacialis*; Hunter et al. 1992) or EPCs (white stork, *Ciconia ciconia*; Tortosa & Redondo 1992) are rare or absent. Moreover, in tree swallows there is no significant effect of breeding density on their levels of paternity (Dunn et al. 1994).

Relatively low EPC rates and even lower EPFs suggest that opportunities for sperm competition (sensu Parker 1970) are rare in the lesser kestrel. This idea is supported by the observation that paired females rejected EPC attempts. Like most raptorial species, female lesser kestrels are larger than males, dominate the males in agonistic encounters (Negro et al. 1992b) and seem to control the success of the copulation attempts (Negro et al. 1992a).

Paired females may refuse to perform EPCs because the costs of practising a mixed-reproductive strategy are higher than that of

remaining faithful to their mates. In kestrel species (*F. sparverius*: Duncan & Bird 1989; Bortolotti & Iko 1992; *F. tinnunculus*: Village 1990; Palokangas et al. 1992) females apparently choose their mates. Males arrive early in the territories and display (e.g. sky-diving and inspecting nest cavities) to attract a female. Similar courtship behaviour has been observed in the lesser kestrel (F. Hiraldo, J.A. Donázar & J.J. Negro, unpublished data). Once the pair bond is established, the female is progressively more dependent on food provisioning by her mate. Before laying, females increase their weight dramatically at the expense of mate feedings (Donázar et al. 1992). During incubation and early nestling stages, the male provides the bulk of the food. When the nestlings are older, males still deliver half or more of the prey to the nest.

Withdrawal of parental care has been suggested to be a form of male retaliation when females engage in EPCs (Trivers 1972; Birkhead & Møller 1992; Whittingham et al. 1993). The extreme dependence of male provisioning in kestrels and other raptors (Newton 1979) could induce the females to remain faithful after making their initial choice. This hypothesis could explain why extra-pair paternity levels were low in our study.

The question then remains as to why lesser kestrels and other well-studied raptors (Møller 1987; Birkhead & Lessells 1988; Shodi 1991; Holthuijzen 1992) copulate at high rates over extended periods. In the near absence of sperm competition, frequent copulation, especially outside the female's fertile period, can hardly be explained as a paternity assurance tactic by the males (Negro et al. 1992a; Petrie & Hunter 1993; Lifjeld et al. 1994).

An alternative to sperm competition is the 'male assessment' hypothesis (Fitch & Shugart 1984; Tortosa & Redondo 1992). Male birds may advertise their good condition by performing energetically costly copulations solicited by the females. Such a mechanism might explain the peak in copulation frequency occurring months before laying reported for the lesser kestrel by Negro et al. (1992a). The male assessment hypothesis is supported by the fact that the pair bond of lesser kestrels sometimes breaks up early in the breeding season (J. L. Tella, unpublished data). None the less, the break-up of pair bonds in lesser kestrels (J. J. Negro, unpublished data) as well as in other birds (Rowley 1983) usually takes place between breeding seasons and not within breeding seasons. The 'male assessment' mechanism proposed by Tortosa & Redondo (1992) views copulations as part of an honest signalling system. A general problem associated to signalling systems is, however, that they may be open to cheating (i.e. if the males diverted energy from other activities at the expense of deteriorating their condition; Henderson et al. 1995), and cannot be used reliably by the females (Simmons 1988).

As an alternative to the male assessment mechanism, a high copulation frequency outside the fertile period could have a yet undetermined pair-bond function (Møller 1987). Further research is needed to determine whether early copulations might help pair members to become sexually functional, and thus accelerate the start to breeding. A decline in nest succcess with laying is widespread in birds (Newton 1979), and any mechanism favouring an early breeding should be adaptive.

Yet another hypothesis is that copulations act as a form of mate guarding by reducing the availability of an individual to other potential mates (Petrie & Hunter 1993). This idea might account for the early peak of copulations in the lesser kestrel, although the specific predictions of the model need to be tested (Petrie & Hunter 1993).

The two cases of intraspecific brood parasitism that we report here are the first observed in the lesser kestrel. Eggs in 52 clutches had been marked and one new egg in two nests appeared well after clutch completion. The frequency of parasitized broods inferred from the nest visits (3.8%, $N=52$) was, however, lower than that revealed by DNA fingerprinting (7.4%, $N=27$). The discrepancy may arise from the difficulty of detecting foreign eggs. Even if nest visits are frequent, brood parasitism may be easily overlooked without molecular techniques (Petrie & Møller 1991; Meek et al. 1994).

We were unable to identify the parasitic females; thus we cannot tell whether they had lost their nests or whether they had viable nests of their own. Nest predation is, however, frequent in the population (Tella et al., in press), and some females may have lost their nests before clutch completion and opted to finish egg laying in other nests, as occurs in the European starling (Feare 1991).

In conclusion, the incidence of EPFs in the lesser kestrel was low compared to other

socially monogamous bird species (Birkhead & Møller 1992). The EPC rate in a previous study overestimated the extent of EPFs, although the difference was small and could be influenced by our sample sizes or because copulatory behaviour and paternity were studied in different populations. The high pair copulation rate of lesser kestrels and other birds is not satisfactorily explained by sperm competition. Frequent copulation in those species has a yet unknown function deserving further study.

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