

Male-biased sex of extra pair young in the socially monogamous Red-backed Shrike *Lanius collurio*

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Abstract. Females of many socially monogamous bird species engage in — or even actively seek — copulations outside their social pair bond. However, in socially monogamous birds with low breeding abundance, such as the Red-backed Shrike, extra-pair paternity (EPP) was thought to be an exceptional and random incident. Drawing on samples collected in an unusually dense Red-backed Shrike population in the Czech Republic, we show through DNA microsatellite typing that among 65 chicks from 15 nests, 10 individuals (26.5%) had been sired by males other than the nest-attending social mate. All 10 extra pair young were of male sex. In all cases, genetic fathers of extra pair young stemmed from neighbouring territories. Extra pair fathers had significantly longer tarsi than social mates, indicating that female choice was a function of age-class dependent male body size. Our findings support sex allocation theory, which suggests that promiscuous females mating with higher quality males should produce mostly sons.

Key words: Red-backed Shrike, *Lanius collurio*, extra pair paternity, sex ratio, secondary sexual character, DNA microsatellite

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The existence of extra pair paternity (EPP) arising from extra pair copulations (EPCs) has been demonstrated in numerous socially monogamous songbird species (for review see Griffith et al. 2002). While the evolutionary advantage of promiscuity can be easily understood from the male perspective, the benefits to females are less clear. It is generally assumed that females may gain indirect genetic benefits from EPP through increased offspring heterozygosity or through the potential to produce sons of higher genetic quality (Petrie & Kempenaers 1998, Griffith et al. 2002). Further, according to the "sexy son" hypothesis (Weatherhead & Robertson 1979), females should preferably mate with males whose genes will produce male offspring with the best chance of reproductive success (Wedell & Tregenza 1999). If so, females engaging in EPCs with high quality

mates should invest more into the production of sons, since male offspring would receive a relative advantage from inheriting paternal traits (Leech et al. 2001).

Despite the sheer quantity of studies devoted to avian mating systems, the causes of variation in the frequency of EPP between species and populations remain elusive (Petrie & Kempenaers 1998). In some socially monogamous bird species, such as Shrikes (Laniidae), EPCs occur regularly but do not necessarily lead to EPP. For instance, in the Lesser Grey Shrike *Lanius minor*, females frequently engage in EPCs after courtship feeding (Carlson 1989, Valera et al. 2003, Tryjanowski & Hromada 2005), but remain genetically faithful to their social mate, as has been revealed by genetic fingerprinting of offspring (Valera et al. 2003). In the closely related Red-backed Shrike, there is to

date only one genetic estimate of the level of EPP, which is based on a very small sample size (EPP among nestlings, 5.26%, $n = 19$; Fornasari et al. 1994).

The level of EPP in a given species is likely to depend on demographic as well as life-history attributes, including breeding abundance, length of the reproductive season and number of broods per season (Petrie & Kempenaers 1998, Griffith et al. 2002). Any biological inference drawn from estimates from single study sites and years (or averaged EPP estimates based on small sample sizes) must be handled with caution, since the frequency of EPP may vary in a density-dependent manner rather than reflect a predisposed species-specific feature of the mating system (for the Pied Flycatcher *Ficedula hypoleuca*, see Lubjuhn et al. 2000). Because Red-backed Shrikes occur in small (and continually declining) numbers, the low frequency of EPP reported so far may simply be due to reduced encounter probability rather than reflect a low intensity of sexual selection in this species.

There is growing awareness that individual behavioural decisions may impact the genetic structure and demography of populations and are thus relevant to conservation and management issues (Quader 2005). Threatened species apparently experience more intense post-mating sexual selection than less threatened species (Morrow & Pitcher 2003). At the population level, habitat heterogeneity is expected to promote polygyny, since females may directly benefit from producing offspring with males occupying territories of higher quality (polygyny threshold hypothesis, Verner & Wilson 1966; see Quader 2005). However, if populations reach low densities, the encounter probability of males and females decreases, which reduces mating opportunities and restricts females from selecting among a wide variety of males and upgrading mating decisions (Quader 2005). As a consequence, females may reduce their investment into offspring or may even decide not to breed at all, given the limited possibilities to increase their fitness. Thus, ongoing population declines in small and fragmented populations may be augmented through the intensity of sexual selection (Quader 2005). A further population genetic consequence of sexual selection and extra-pair paternity is the potential bias in the sex ratio of extra-pair young (EPY). To our knowledge, there is only one ornithological study documenting a male-biased sex ratio among EPY (Kempenaers et al. 1997).

The aim of the present study was to assess the EPP in high density population of Red-backed Shrike.

The study plot (15 km²) was situated in South Bohemia, north-east of the town of Písek, Czech Republic (49°19'N, 14°15'E) where adult and nestling Red-backed Shrikes have been colour ringed since 1989. Breeding site fidelity in this population is generally high (for details see Šimek 2001) so that intensive nest searches and observation of colour ringed individuals gave a detailed map of territory ownership and social pair bonds within the study area. Population density averaged 3.5 pairs/km², which is among the highest breeding densities documented in the Red-backed Shrike (see Kuźniak & Tryjanowski 2003). Brood size ranged from two to five nestlings (mean \pm SD = 4.0 ± 1.0 , $n = 15$ nests), which lies within the range of values published elsewhere (review in Kuźniak & Tryjanowski 2003).

Adult birds were sexed and aged according to plumage characters described by Svensson (1992). In addition, we took basic morphometrical measurements, including the length of the right wing (maximum chord, to the nearest 0.5 mm, using a metric ruler), the length of the right tarsus (to the nearest 0.05 mm, using callipers) and body mass (to the nearest 0.1 g, using a spring balance). All morphometrical measures were conducted by one and the same person (JŠ).

The paternity analysis presented in this paper was based on DNA samples collected in a single breeding season (1998). 5–50 μ l blood was taken from the brachial vein in adult birds and nestlings. Nestlings were sampled and ringed from day 7 onwards (day 0 = hatching day). Blood samples were collected in 70 μ l capillary tubes, cooled on ice in the field and stored in a freezer at -18°C on the same day. Social parentage of each brood was determined by observing nest attendance of individually colour ringed adults during chick rearing. DNA was extracted from blood samples using a phenol/chlorophorm protocol (Milligan 1998). Individual birds were genotyped using four polymorphic microsatellite loci (*LS1*, *LS2*, *LS3*, *LS4*), which were originally developed for the Loggerhead Shrike *Lanius ludovicianus* (Mundy & Woodruff 1996), but amplify in the Red-backed Shrike (Pustjens et al. 2004). For each PCR step, a total volume of 20 μ l contained 0.5 units Taq DNA Polymerase (Invitrogen), 1 x PCR buffer (20mM Tris-HCl (pH = 8.4), 50 mM KCl), 0.2 mM dNTPs each, 1.5 mM MgCl₂, 0.5 μ M primers each and 2 μ l

of template DNA. PCRs were run on GeneAmp 9700 Thermocycler (Applied Biosystems). The PCR profile consisted of an initial denaturing step at 94°C for 3 min, followed by 35 cycles of 94°C for 30s, X°C for 1min (X = 47°C for *LS1*, 50°C for *LS2*, 53°C for *LS3* and 55°C for *LS4* loci), and 72°C for 1 min. The cycle was terminated with a step of 72°C for 7 min followed by 4°C. The bands were visualized in 6% polyacrylamide gels stained by SYBR® Green (Molecular Probes).

Nestlings were sexed according to a universal PCR method based on the amplification of CHD genes located on the avian sex chromosomes (Griffiths et al. 1998). Adult birds could be sexed by their plumage characters, yet information was confirmed by PCR based methods. PCR amplifications were run in a total volume of 20 μ l. Each reaction contained 0.5 units Taq DNA Polymerase (Invitrogen), 1x PCR buffer (20mM Tris-HCl (pH = 8.4), 50 mM KCl), 0.2 mM dNTPs each, 1mM MgCl₂, 0.5 μ M primers each and 2 μ l of template DNA. The PCR profile consisted of an initial denaturing step at 94°C for 1 min, followed by 30 cycles of 94°C for 30s, 48°C for 45s, 72°C for 45s, and terminated by 94°C for 30s, 48°C for 1 min and 72°C for 5 min. Bands were visualized in 2% agarose gels stained with SYBR® Green (Molecular probes). Because the number of sampled broods and applied microsatellite loci (n = 4) was relatively small, we conducted paternity assignments only for those broods (n = 15) where both social parents had been genotyped (Kasumovic et al. 2003, Kalinowski et al. 2007). Offspring were defined as extra-pair young (EPY) when two or more loci deviated from the social male. The likelihood of genetic paternity was established using the software package CERVUS 3.0 (Kalinowski et al. 2007). Statistical analyses were performed with SPSS 12.0. All tests were two-tailed with a significance level of $p < 0.05$. Results are presented as means \pm SD.

Extra pair young (EPY) were detected in 6 (40%) of the 15 analysed broods, each containing one to three (1.7 ± 0.8 , n = 6) EPY. Among the 65 sampled nestlings, 55 (84.6%) had been sired by social mates, whereas 10 (26.5%) resulted from EPCs of 3 males. In all cases, genetic fathers of EPY stemmed from neighbouring territories. All individuals observed outside their own territories (intruders) were males. This pattern differed significantly from random (n = 7, binominal sign test, $p = 0.016$).

The sex ratio of offspring shared among social

pairs was 1.39 (32 males versus 23 females). All 10 nestlings sired by extra pair males were of male sex. Thus, the net sex ratio of all sampled nestlings accumulated to 1.83. The contrast in sex ratio between offspring among social partners and those sired by extra pair males was statistically significant (χ^2 with Yates correction = 4.792, df = 1, $p = 0.029$).

Extra pair fathers had significantly longer tarsi than faithful males (24.30 ± 0.10 versus 23.22 ± 0.40 mm, sample size for extra pair males and faithful males was 3 and 14, $t_{14} = -8.67$, $p < 0.0001$). Interestingly, in all three cases, extra pair young were sired by genetic fathers of the females' social partner.

Extra-pair paternity is widespread among birds, and illegitimate offspring have been found in approximately 90% of the examined species (reviewed by Griffith et al. 2002). Our study adds to the plethora of studies reporting significant genetic promiscuity in a socially monogamous songbird. In contrast to the low level of EPP found in a Red-backed Shrike population in the Italian Alps (Fornasari et al. 1994), we show that EPP can reach higher levels in this species. Furthermore, compared with other passerines with a similar mating system, i.e. social monogamy (16% EPP averaged across 61 species; value derived from Appendix 1 in Griffith et al. 2002), our EPP estimate for the Red-backed Shrike lies exactly in the range of EPP estimates in this group of birds. Our estimate of the frequency of EPP in the Red-backed Shrike lies above the estimates found in all other species of Laniidae studies so far. Using multi-locus DNA fingerprinting, Yamagishi et al. (1992) found that 10 of 99 (10%) nestlings of the Bull-headed Shrike *Lanius bucephalus* had been sired by extra pair males. In the Loggerhead Shrike eight offspring from five families (4% of offspring) were sired by extra pair males (Etterson 2004). In the Lesser Grey Shrike, although females frequently engaged in EPC, EPP has so far not been genetically detected (Valera et al. 2003).

Our result questions the previous view that the Red-backed Shrike is strictly monogamous, which was based on observational data alone, with only one case of social polygyny found in this species (Olsson 2001). This had lead to the assumption that either females are unwilling to accept EPCs, or that males cannot monopolize the territorial resources needed to attract more than one female. Our study shows that female Red-backed Shrikes do accept EPCs from owners of neighbouring territories.

There was a tendency towards longer tarsi in males mated to females outside their social pair bond. In the Red-backed Shrike, old males generally have longer tarsi than males of a younger age class (Jakober & Stauber 1989, Tryjanowski & Yosef 2002, Tryjanowski & Šimek 2005). Thus, the association between male tarsus length and EPP may reflect that females choose males according to their age. This age-class dependent female choice is further supported through cases where females reproduced with fathers of their social mates. Together, this matches the general pattern seen in songbirds where older males are favoured by female choice (e.g. Lubjuhn et al. 2000, Griffith et al. 2002, Kleven et al. 2006).

The quality of extra pair males may also explain why the sex-ratio of their offspring was male-biased. Theory predicts that cryptic, post-copulatory female choice should favour the production of "sexy sons" after mating with males of higher quality, through which they may gain an indirect fitness advantage (see introduction). Previous behavioural observations such as strong defence responses at territory borders by males (Durango 1956, Fornasari et al. 1992, 1994), as well as intensive mate guarding of receptive females (Jakober & Stauber 1994), support the high probability of EPP resulting from EPCs in the Red-backed Shrike. Our study underpins these suggestive observations and calls for further detailed studies on mate choice, sexual selection and genetic variation in Red-backed Shrike populations (cf. Pustjens et al. 2004).

In conclusion, our study shows that the level of detected EPP in the Red-backed Shrike is higher than previously reported. Since the Red-backed Shrike is critically endangered in many parts of Europe, and has vanished in the British countryside, its prospects for the future are concerning. With increasing habitat fragmentation and locally decreasing habitat quality, the relatively high level of sexual selection through female choice found in this species may limit overall fecundity. Female choice could potentially drive populations into evolutionary traps, since the cues upon which females base reproductive decisions may fall below threshold, leading to maladaptive mating decisions or even complete reproductive failure (cf. Quader 2005).

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STRESZCZENIE

[Nieproporcjonalnie duży udział samców wśród piskląt gąsiorka]

Samice wielu gatunków monogamicznych angażują się, a nawet aktywnie poszukują, kopulacji pozapartnerskich. Jednakże, u gąsiorka, socjalnie monogamicznego gatunku żyjącego w stosunkowo niewielkich zagęszczeniach wykazywano bardzo niski (i raczej tylko okazyjny) udział kopulacji pozapartnerskich. Na podstawie materiałów zebranych w środkowych Czechach i późniejszych analiz genetycznych, w izolowanej populacji tego gatunku stwierdzono, że 10 piskląt (26.5%, spośród zbadanych 65 piskląt pochodzących z 15 gniazd) miało innego genetycznego ojca, niż partner socjalny samicy. We wszystkich przypadkach ojcami tych piskląt były samce z sąsiednich terytoriów.

Samce, które posiadały zarówno potomstwo z własną samicą, jak i dodatkową partnerką innego samca, charakteryzowały się dłuższym skokiem, co może wskazywać, że w kopulacje pozapartnerskie angażują się starsze, bardziej doświadczone samce. Stosunek płci wśród piskląt z lęgów pochodzących od partnerów socjalnych wynosił 1.39 (32 samców vs. 23 samice), zaś spośród 10 piskląt mających innych genetycznie ojców, niż socjalny partner ich matki, wszystkie 10 było samcami.