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Communal Breeding in the European Starling: Evidence from DNA Fingerprinting

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The mating system and reproductive strategies of the European Starling (*Sturnus vulgaris*) recently have received considerable attention. Both polygyny and intraspecific nest parasitism have been regularly recorded in several populations (Merkel 1980, Lombardo et al. 1989, Pinxten et al. 1989, 1991a, b, Pinxten and Eens 1990, Romagnano et al. 1990). Although cooperative breeding has been reported in several African starlings (Wilkinson 1982, Craig 1983, 1987), it has only rarely been recorded in the European Starling (Stouffer et al. 1988). Here, we report a case in which two female European Starlings laid eggs in the same nest and cooperated in feeding the nestlings, while only one male was observed feeding the nestlings. Using DNA fingerprinting, we could confirm that the three adults attending the nest were the biological parents of the nestlings, and that the male was simultaneously bigamous.

The nesting occurred in 1992 in a nest box colony in Zoersel, near Antwerp, Belgium. This colony has been monitored since 1985 as part of an ongoing study of reproductive strategies in European Starlings (see Pinxten et al. 1989, 1991a, b). During 1985–1990, nearly all breeding adults were banded with metal bands of the Belgian Ringing Scheme and individually color marked with wing tags. Age (second-year bird vs. older bird) was determined by measuring (to nearest millimeter) the length of iridescence of the throat feathers (Delvingt 1961). The behavior of the birds was recorded throughout the breeding season. Polygyny and intraspecific nest parasitism occurred regularly with frequencies ranging from 20 to 40% and 0 to 37%, respectively (Pinxten et al. 1989, 1991a, b, Pinxten and Eens 1990). From 1991 onwards fieldwork in this colony was restricted to routine inspections of the nest boxes to collect breeding data.

During a routine inspection of the nest boxes on 21 April 1992, one (nest box 3) was found to contain a nest with 10 starling eggs. Clutch size in the other nests in the colony ranged from five to seven eggs, and was on average 5.9 ± SD of 0.6 (n = 15). During 1985–1991, large clutches (8 or more eggs) comprised only 2% (4/187) of all clutches in the colony. Inspection of the eggs suggested that the clutch was laid by two different females in that five eggs of the clutch were darker than the others and appeared to be smaller. In the European Starling, interclutch variation in eggs is considerably greater than intraclutch variation (Greig-Smith et al. 1988), and the eggs of certain females may be distinguishable on the basis of size, shape and color (Feare 1991). All 10 eggs were measured (maximum length and breadth) to the nearest 0.1 mm with a Vernier caliper, and an index of volume (V) calculated using

$$V = KLB^2, \quad (1)$$

where K is a scaling constant (see Greig-Smith et al. 1988), L is length, and B is breadth. The volume index differed significantly between the five light-colored and the five dark-colored eggs (6.87 cm^3 ± 0.07 vs. 6.29 cm^3 ± 0.15; Mann-Whitney U-test, U = 0.00, $P < 0.01$), supporting the suggestion that the two females each laid five eggs in the nest. As starling nests in the colonies we study often contain the eggs of more than one female due to intraspecific nest parasitism (Pinxten et al. 1991a, b), it was first thought this was a case of intraspecific nest parasitism, where the nest was parasitized five times (cf. Evans 1988).

As the date of clutch initiation and completion was unknown, the nest box was inspected daily from 21 April onwards until hatching. On 29 April (1330 standard time), the nest box contained two newly hatched nestlings that had hatched from two light-colored eggs and eight unhatched eggs. The following day (1245) it contained eight nestlings and two unhatched eggs (one light-colored and one dark-colored). On 2 May (no check made on 1 May), the dark-colored egg had hatched, while the light-colored one had not. These observations suggest that: (1) the female that laid the light-colored eggs started laying first; and (2) the time interval between clutch initiation of the two females must have been only one or two days (starlings usually start incubation the day before clutch completion; Feare 1984, but see Meijer 1990). When examining the content of the unhatched light-colored egg, we found no trace of embryonic development, suggesting it was not fertilized.

Between 5 and 7 May, three adults were captured with automatic nest-box traps (see Pinxten et al. 1989) when feeding nestlings. On 5 May, a female (female 1) that had hatched from a first brood in the colony in 1990 was captured. On 6 May, we observed two unmarked birds, a male and a female, feeding the nestlings, suggesting that this was a case of communal breeding instead of intraspecific nest parasitism. We captured the male that day. On 7 May, when female 1 was observed again feeding the nestlings, the second female (female 2), was captured when feeding the nestlings. All three adults involved in the communal breeding were older birds. On 12 May, we recorded feeding frequencies during 1 h. Female 1 fed the nestlings three times, female 2 six times, and the male twice. Later observations showed that all

three adults continued to feed the nestlings until fledging. We do not know whether both females also cooperated in incubating the eggs, and/or whether the male assisted in incubation, since we did not monitor the nest during incubation. However, it is unlikely that a single European Starling can effectively incubate 10 eggs (see Pinxten et al. 1993a). The only other nest containing 10 eggs (due to dumping of three parasitic eggs during laying period) we ever recorded in the colony was deserted two days after clutch completion. Moreover, eight eggs was the maximum number of eggs a starling pair in the colony ended up successfully incubating after being parasitized, suggesting that at least two of the three adults involved in the communal breeding must have participated in incubation. We never observed a second male attending the nest, suggesting the male was simultaneously bigamous (with the time difference between clutch initiation of the two females being less than two days). During the total of about 350 min that we monitored the nest, we also never observed aggressive interactions between the two females.

All nine hatchlings fledged. The mean number of fledglings of the 15 other first clutches in the colony was 5.6 ± 0.5 (range 4 to 6), excluding a brood that failed completely due to predation. The average mass of the nine nestlings at 15 days of age was 73.55 ± 3.21 g (range 66–77 g). This was significantly lower than that of the nestlings in nest box 1 (77.33 ± 2.25 g, range 73–79 g, $n = 6$; $U = 7.0$, $P = 0.017$) and nest box 12 (80.66 ± 3.67 g, range 76–85 g, $n = 6$, $U = 1.5$, $P = 0.0008$), which had hatched on the same day.

Our DNA fingerprinting methods were as described in Kempenaers et al. (1992). DNA was extracted from blood (100–500 μl from brachial veins of adults and six- to seven-day-old nestlings), completely digested with the restriction enzyme *Hinf*I, separated by agarose gel electrophoresis, transferred to a nylon membrane and hybridized with the radiolabelled minisatellite probe 33.15 (Jeffreys et al. 1985a). For parentage assignment, we followed the procedure described by Pinxten et al. (1993b). The putative parents were considered as the true genetic parents whenever the nestling fingerprint contained only one or no novel bands. Single novel bands were regarded as resulting from mutation.

In our assessment of parentage using DNA fingerprinting, we were able to score on average 17.2 bands per fingerprint, with a range of 14 to 22. Band-sharing coefficients (twice the number of shared bands divided by sum of bands for the two individuals; Wetton et al. 1987) for the three adults averaged 0.26 ± 0.01 (Table 1). This value is similar to that for unrelated starlings in our population (0.21 ± 0.06, calculated as the band sharing proportions for 15 pairs of parents) and indicates that the three adults involved in the communal breeding were not related to each other. The use of band-sharing proportions as an index of genetic relatedness (see also Table 1) is based on the assumption that bands are inherited independently. This assumption can best be tested by examining pairs of bands transmitted to offspring in a large family of 10 or more offspring (Burke et al. 1989). Unfortunately, such a large family did not exist in our samples, but as emphasized by Westneat (1990) and Amos et al. (1992), uncertainties about the genetics of fingerprint bands do not prevent use of patterns to examine parentage. Moreover, the probe 33.15, which we used in this study, has proved to detect randomly dispersed DNA fragments with minimal allellism and linkage in combination with the restriction enzyme *Alu*I in starlings (Pinxten et al. 1993b). However, in this study we used the restriction enzyme *Hinf*I, and independent segregation of DNA fragments has in fact to be tested for each enzyme/probe combination to be used (Hanotte et al. 1992). Until a proper segregation analysis can be performed, we have to assume minimal allelism and linkage.

As the minimum number of bands scored in an offspring was 14 (Fig. 1), the probability of false inclusion of a misassigned pair, as might occur due to intraspecific nest parasitism is very small ($P < 0.000011$; Burke et al. 1989). Also, we scored at least five paternal bands in each offspring, from which we obtained a maximum value for the probability of false inclusion of an unrelated male of 0.001 (Jeffreys et al. 1985b, Davies et al. 1992). Mothers were initially assigned by the presence of diagnostic bands in each nestling (range 5–11, $\bar{x} = 7.7 \pm 2.3$). Eight of the nine nestlings did not show any novel bands in their fingerprints, while one nestling (nestling 9; see Fig. 1, Table 1) showed only a single novel band. Therefore, the putative parents were considered as the true genetic parents (cf. Burke et al. 1989, Pinxten et al. 1993b). The genetic analysis revealed that female 1 was the mother of five of the nine nestlings, female 2 of the remaining four nestlings, and that the male fathered all nine nestlings (Fig. 1, Table 1). These results confirm the behavioral observations that: (1) both females were actually mated to the male in question; and (2) each female contributed five eggs to the clutch.

This is the first time we observed and identified two females laying in the same nest where both females were paired to the male owner and, subsequently, cooperated in feeding nestlings. Vanvinckenroge (1968) also observed two female European Starlings breeding in a large Tawny Owl (*Strix aluco*) nest box in Belgium. However, in this case the two females laid their eggs in separate nests. Stouffer et al. (1988) were the first to report a case in which two European Starling females laid eggs in the same nest and cooperated in incubating the eggs and in feeding the nestlings in three breeding attempts. In this case of communal nesting in North America, the same male was observed feeding the young in two of the three broods.

Close kinship is often found among birds cooperating at the same nest (Brown 1978). The genetic anal-

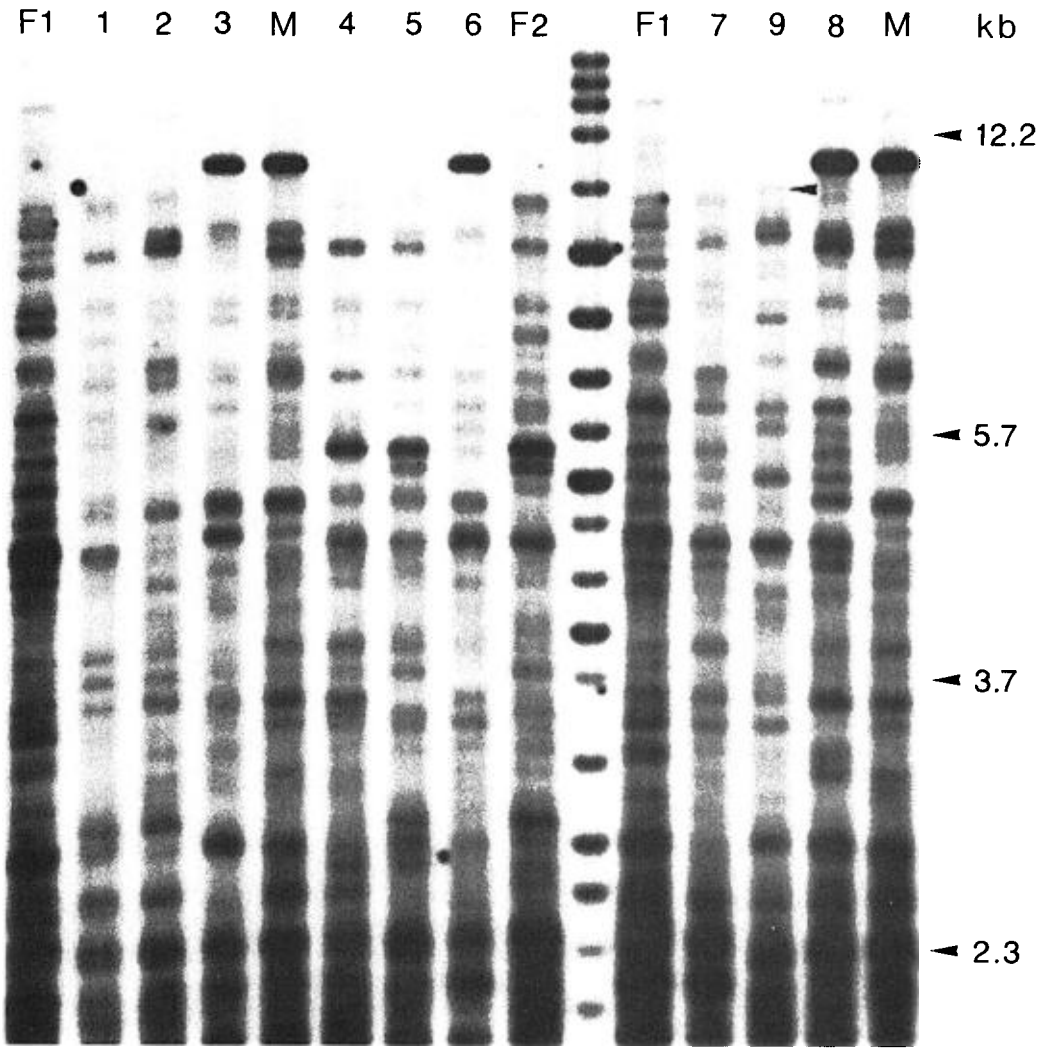

Fig. 1. DNA fingerprints from three adult European Starlings (M = male, F1 = female 1, F2 = female 2) involved in communal breeding, and nine offspring (1–9) in nest. Fingerprints generated from *Hinf*I-cut DNA hybridized with multilocus probe 33.15. M is father of all nine nestlings. F1 is mother of nestlings 3, 6, 7, 8 and 9, and F2 is mother of nestlings 1, 2, 4 and 5. Nestlings 1–8 did not show novel bands in their fingerprint (i.e. bands that had no co-migrating counterpart in fingerprints of either or both assigned parents), while nestling 9 had a single novel band (indicated by solid arrowhead). Scale to right indicates fragment sizes in kilobases, determined by comparing their migration distance with that of length markers of known sizes (Analytical Marker System, Promega). One lane containing such length markers is situated between two lanes with the fingerprints of F2 and F1.

ysis revealed that the three adults involved in the communal breeding were unrelated.

Simultaneous bigamy has been recorded during the first brood-laying period in the Zoersel colony (11% of 26 observed cases of bigamy during 1985–1989; see Pinxten and Eens 1990, Pinxten et al. 1993b). Behavioral observations revealed that these cases of polygyny are due to a lack of unmated males at that time (see Pinxten and Eens 1990). However, the current case is the only one of simultaneous bigamy we observed in which both females bred in the same nest. Stouffer et al. (1988) suggested that the cases of communal breeding they documented in North America may have been the result of a lack of suitable nest sites in their colony. However, this is unlikely to be the cause in our colonies, since more than one-half of the nest boxes were unoccupied during the first-brood laying period in 1992, including several in the

TABLE 1. Band-sharing coefficients (see text) for all pairwise combinations of three adult European Starlings (M = male, F1 = female 1, F2 = female 2) and nine offspring in nest. Values for nestlings and their assigned parents are in bold and are close to expected for parent-offspring pairs (0.59; Jeffreys et al. 1985b). Nestlings 1–8 did not show novel bands in their fingerprint, while nestling 9 had a single novel band (see Fig. 1).

Bird	F1	F2	1	2	3	4	5	6	7	8	9
M	0.26	0.26	**0.55**	**0.55**	**0.55**	**0.61**	**0.51**	**0.57**	**0.51**	**0.64**	**0.43**
F1		0.25	0.23	0.21	**0.49**	0.30	0.31	**0.50**	**0.61**	**0.66**	**0.68**
F2			**0.53**	**0.59**	0.18	**0.65**	**0.80**	0.27	0.33	0.22	0.16
1				0.58	0.36	0.47	0.53	0.40	0.40	0.41	0.43
2					0.29	0.49	0.54	0.30	0.36	0.33	0.22
3						0.29	0.29	0.60	0.43	0.51	0.43
4							0.65	0.39	0.45	0.44	0.37
5								0.44	0.44	0.31	0.26
6									0.56	0.54	0.47
7										0.58	0.47
8											0.56

immediate vicinity of nest box 3. As the behavior of the birds was not recorded during the prelaying period, we can only speculate as to why both females shared the same nest box and male.

The fact that all nine hatchlings fledged shows that in this case there was no reduction in fledging success due to the communal breeding. However, we cannot exclude the possibility that the female that started laying first may have had her first egg(s) removed by the second female, as female starlings have been demonstrated to remove eggs added to their nest before they start laying themselves (Stouffer et al. 1987, Pinxten et al. 1991c). Also, the nestlings weighed significantly less than those in broods hatching on the same day, which may have resulted in increased mortality after fledging (see Pinxten and Eens 1990).

Compared to the average breeding success of simultaneous bigamous males in the Zoersel colony during 1985–1989 (6.3 ± 5.68, $n = 3$; when excluding one male whose two females both deserted their brood, one during the incubation and one during the nestling stage, 9.5 ± 2.12, $n = 2$), the male did not seem to suffer strongly reduced breeding success due to the communal breeding of his females. Male European Starlings usually guard their mates intensively during their presumed fertile period to protect their paternity (Power et al. 1981, Pinxten et al. 1987), and extrapair copulations have been observed infrequently in the Zoersel colony (Eens and Pinxten 1990, Pinxten et al. 1993b). Nevertheless, DNA fingerprinting revealed that 10% of nestlings in the Zoersel population are the result of extrapair fertilization (Pinxten et al. 1993b). As the fertile period of the two females must have overlapped considerably, the male potentially could have been unable to guard both females efficiently and might have lost some paternity. The DNA fingerprinting, however, revealed that the male was the father of all nine nestlings. This suggests that the male in question may have been a high-quality male and that both females may not have tried to engage in extrapair copulations with another male (cf. Kempenaers et al. 1992).

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