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NO EVIDENCE FOR EFFECTS OF BREEDING DENSITY AND MALE REMOVAL ON EXTRAPAIR PATERNITY IN THE PIED FLYCATCHER

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Abstract.—Molecular methods have brought new insight to the study of mating patterns. Extrapair fertilizations (EPF) have proven to be widespread among bird species irrespective of social relationships. Ecological factors, such as breeding density and synchrony, have been suggested as contributors to variation in EPF rates. Absence of a male during the female fertile period may also increase the probability of EPFs. In this study, we examined experimentally whether breeding density and male absence before egg-laying influence the EPF rate in Pied Flycatchers (*Ficedula hypoleuca*). DNA fingerprinting revealed that 4.4% (10/225) of nestlings were the result of EPFs and 7.0% (3/43) of broods included at least one offspring sired by a male other than that attending the nest. We did not find any evidence of higher EPF rate with higher breeding density as stated by the density hypothesis. Contrary to expectation, EPFs were more frequent, though not quite statistically significantly, at low breeding density: all three EPF nests were found in low-breeding-density areas. There was no evidence that EPF rate was affected by a 4 h male removal. In all cases of EPF, an old female was mated with a yearling male, which suggests that older females mated with browner yearling males may have adjusted their initial mate choice through EPCs to acquire indirect genetic benefits. Pied Flycatcher females may be constrained to accept a mate of lower than preferred quality, especially at low breeding density, due to the cost of searching for alternative males. *Received 1 November 1999, accepted 9 August 2000.*

Molecular methods have revolutionized the study of mating patterns. Early descriptions of mating systems have usually addressed only the social component, but contemporary studies are also able to address the genetic component of mating systems. Those two components may be interrelated, but the correlation does not necessarily have to be strong. Behavioural studies and recent use of molecular techniques have revealed that extrapair copulations (EPC) and fertilizations (EPF) are widespread phenomena among bird species irrespective of the social mating pattern characterizing a species (Birkhead and Møller 1992). Variation in the rate of EPF is great and can at least partly be attributed to differences among species, but there is also considerable variation within species (Birkhead and Møller 1992).

Ecological factors, such as breeding density and synchrony, have been suggested as contributors to variation in EPF rate (Westneat et al. 1990, Birkhead and Møller 1992). According to the density hypothesis, males have more opportunities to copulate with extrapair females in denser populations (Westneat et al. 1990, Birkhead and Møller 1992). Assuming that EPCs translate to EPFs, the EPF rate should increase with increasing density, and that hypothesis has some support. In a recent review, Westneat and Sherman (1997) found that the EPF rate was in most cases positively associated with density within species. However, comparison among species did not reveal any association between breeding density and EPF rate (Westneat and Sherman 1997).

The Pied Flycatcher (*Ficedula hypoleuca*) is a small migratory passerine bird that readily breeds in nest boxes. Most males mate monogamously but many males establish a second territory, and about 15% (range 3 to 39%) of the males successfully attract a second female if

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additional nest sites are available (Lundberg and Alatalo 1992). The second territory is spatially separated from the first one, usually by a distance of about 200 m (Lundberg and Alatalo 1992). Males often establish a second territory just after the onset of egg laying by the first female (von Haartman 1956, Silverin 1980, Lifjeld et al. 1991, Rätti and Alatalo 1993). However, many males leave their first territory before egg laying and, thus, are absent at least temporarily from their territory during the fertile period of their primary mate. Male removal experiments in the Pied Flycatcher and other species have shown that absence of the male during the female's fertile period increases the probability of EPCs and EPFs (Björklund and Westman 1983, Møller 1987, Birkhead et al. 1989, Björklund et al. 1992, Westneat 1994).

Several studies have reported that EPCs occur in the Pied Flycatcher (von Haartman 1951, 1956; Björklund and Westman 1983, Alatalo et al. 1987). Alatalo et al. (1987) observed the EPC rate to be 29%. Five studies of EPF in the Pied Flycatcher using molecular techniques show that the EPF rate varies considerably (4–24% of nestlings, 15–43% of broods; Lifjeld et al. 1991, Gelter and Tegelström 1992, Ellegren et al. 1995, Rätti et al. 1995, Brün et al. 1996). In the present study, we investigated experimentally whether breeding density and male absence before egg laying influence the EPF rate in the Pied Flycatcher. We predicted that the EPF rate would increase both with increasing density and male removal. Also, we examined whether breeding density may explain the variation in Pied Flycatcher EPF rate among different studies.

METHODS

Field procedure.—Field work was carried out in the vicinity of the Konnevesi Research Station (62°37'N, 26°20'E) in central Finland during 1992. The study area consisted of coniferous forest mixed with birches. Nest boxes were erected about 1.6 m above the ground.

We manipulated breeding density using two kinds of nest box distributions at five study plots and along four roads. We had five study plots with 8–29 nest boxes, in which the mean internest distance was 74 m (range 30 to 200 m, $n = 51$). That corresponds to a breeding density of 1.8 pair/ha and was considered high breeding density. The nest boxes distributed along four different roads (4 to 13 boxes each) were separated by at least 300 m. Those lines of nest boxes had a final internest distance of 564 m (range 300 to 1000 m, $n = 25$) corresponding to a breeding density of 0.03 pairs/ha and were considered as low-breeding-density sites. Forests of the study area are managed and number of natural cavities is low. The presence of Pied Flycatchers breeding in natural cavities was checked by patrolling around occupied nest boxes to observe singing males. We did not find any Pied Flycatchers breeding in natural cavities, but observed some breeding in nest boxes near houses.

We followed the progress of nest building daily to predict onset of egg laying. We grouped nests into quartets (two from high density and two from low density) according to the stage of nest building. Then we randomly chose one nest of either density for male removal. When the nest was completed, we captured the male from that nest by mist net and kept it in a cage for 4 h. During the removal, the male was provided with live mealworms. Initially we had 12 quartets. In one case, we failed to remove the male at low density. Male removals were made one ($n = 13$), two ($n = 6$), or three ($n = 1$) days before the onset of egg laying ($\bar{x} = 1.4$ days) and between 0800–1500 (EST).

We sampled blood from males at the time of the experimental removal and from females during the late incubation period. During the nestling period, we sampled blood from nestlings and control males. We estimated the proportion of brown back feathers of males on the scale 0–100% (a measure for male coloration, see Lundberg and Alatalo 1992 for details). Wing, tail, and tarsus length were measured as well as the body mass of both parents. Birds were aged as yearlings or older on basis of the amount of wear on primaries and outermost greater coverts (Karlsson et al. 1986).

The procedure of grouping nests into quartets removed differences in the distribution of egg-laying dates between treatments (Kolmogorov-Smirnov test: density, $D_{\max} = 0.10$, $P = 0.95$; removal, $D_{\max} = 0.17$, $P = 0.55$). However, we were not able to control fully for differences among individuals belonging to the different treatments. Females in the low-density areas differed morphologically from females that bred in the high-density areas (MANOVA, $F = 2.93$, df = 4 and 36, $P = 0.03$). Univariate statistics revealed that females breeding in the low-density areas had significantly longer wings ($\bar{x} = 79.2$, SD of 1.7 and 77.6 ± 1.6; $F = 9.43$, df = 1 and 39, $P = 0.004$) and tails ($\bar{x} = 51.2 \pm 1.7$ and 49.7 ± 1.7; $F = 8.98$, df = 1 and 39, $P = 0.005$) than females breeding in the high density areas. Male or female morphology did not differ statistically between male removal treatments (MANOVA, $P > 0.10$). Male or female age was not associated with breeding density or male removal (chi-square test, $P > 0.10$ for all)

In three cases, nestlings died before sampling and one nest was removed due to low quality of fingerprint. Thus, in total, we successfully sampled 43 families (86 adults and 225 nestlings). Twenty-three of the nests were at high-breeding density areas and 20 were at low-density areas. Male removal was conducted at 11 nests at high breeding density and 9 nests at low breeding density.

DNA fingerprinting.—For DNA fingerprinting, we collected approximately 80 μL blood from the brachial or tarsus vein by a haematocrit capillary after puncturing the vein. The blood was transferred to polypropylene tubes containing 100 μL TNE buffer (0.15 mM trisodium citrate, 0.15 M NaCl, 0.5 mM EDTA, pH 7.0) and stored in a −20°C freezer. Laboratory work was carried out at the Department of Genetics, Uppsala University. The insert of human minisatellite clone 33.15 (Jeffreys et al. 1985a) was used for DNA fingerprinting. The DNA profiles were run on large gels and the suspected parents were always run on the same gel with offspring to avoid difficulties in determining presence or absence of bands among offspring. For a detailed description of laboratory procedures see Rätti et al. (1995).

Paternity detection.—To assess the paternity from the DNA fingerprints, we identified maternal and paternal bands from acetate sheet overlays (i.e. bands not shared by the two putative parents), and assessed presence or absence of such bands among nestlings of a brood. On average, we scored 15.0 (range 8 to 25) bands per individual. Bands not occurring in DNA fingerprints of the putative parents (hereafter called mismatched bands) should theoretically originate either from mutation or from extrapair parentage (EPF or extrapair maternity). The mutation frequency in avian minisatellite loci has been estimated as between 2 and 11 $\times 10^{-3}$ mutations/locus/meiosis (e.g. Burke and Bruford 1987, Westneat 1990, Gelter and Tegelström 1992, Kempenaers et al. 1992). We therefore expected that only a few mismatched bands would be randomly distributed among nestlings. Extrapair parentage will lead to a higher number of mismatched bands in a nestling than expected from mutation alone.

We also calculated the proportion of bands shared between two individuals (D) by dividing twice the number of shared bands by the sum of bands for the two individuals (Wetton et al. 1987). The mean proportion of shared bands between parents was 0.131 ± 0.070 ($D \pm$ SD, $n = 43$), which gives an estimate for the probability of two individuals sharing a band by chance alone (x, Birkhead et al. 1990). Mean allele frequency was $q = 1 - (1 - x)^{1/2} = 0.068$ (assuming that all alleles are of equal frequency; Jeffreys et al. 1985a). The expected proportion of parental bands shared by an offspring (first order relative) is $D = (1 + q - q^2)/(2 - q) = 0.550$ (Jeffreys et al. 1985b, Burke and Bruford 1987).

The mean probability of false inclusion of a nestling that was a result of intraspecific brood parasitism was very small $P = (1 - (1 - x)^2)^n = 6.8 \times 10^{-10}$ (n = mean number of bands scored per nestling = 15.0, see Burke et al. 1989). The mean probabilities of false inclusion of a non-mother or a non-father were also small, $P = x^m = 3.6 \times 10^{-7}$ and $P = x^p = 2.0 \times 10^{-7}$ (m = mean number of maternal bands = 7.3 and p = mean number of paternal bands = 7.6, see Burke et al. 1989).

Nestlings with fewer than three mismatched bands were considered to be offspring of both the putative parents. Nestlings showing more than the expected number of mismatched bands that could be explained by mutation alone, having only a few bands in common with the putative father, and about half of the bands in common with the putative mother, were regarded as sired by EPF.

Statistical analyses.—Statistical analyses were performed using SPSS for Windows 6.1.3. Two-tailed probabilities are used throughout.

To analyse whether the probability of an individual to be associated with EPF was dependent on a continuous independent variable, we used logistic regression analysis (Trexler and Travis 1993). The logistic regression model is $\ln(p/1 - p) = b$ (independent variable) $+ a$, where p is binomial probability, and b and a are parameters. Model significance was tested by change in deviance (ΔD) with associated change in degrees of freedom which approximates a chi-square distribution.

Some males in this study were polygynous. Thus, the data set includes two nests each from four of the males. In those cases, we included one randomly chosen observation per male into analyses.

RESULTS

DNA fingerprinting.—We found 10 offspring with more than two mismatched bands (range 3 to 10). Those nestlings had low band-sharing coefficients with the male (range 0.000 to 0.235), which suggests that they were the result of either EPF or intraspecific brood parasitism. All of those nestlings shared a large proportion of bands with their putative mothers (range 0.387 to 0.667), and thus intraspecific brood parasitism could be excluded. The EPY were scattered among three nests; 1/6, 3/7, and 6/7 in each. Thus, 4.4% (10/225) of nestlings were the result of EPFs and 7.0% (3/43) of broods included at least one offspring sired by a male other than that attending the nest.

The proportion of bands shared between mothers and their confirmed nestlings was on average 0.558 ± 0.094 (range 0.286 to 0.839, $n = 225$). Band sharing between the nestlings and their confirmed fathers was 0.576 ± 0.091 ($D \pm$ SD, range 0.270 to 0.800, $n = 215$). Both

TABLE 1. Proportion of broods with EPY in different treatment categories in the Pied Flycatcher.

	Density		
	High	Low	Total
Male not removed	0/11	1/9	1/20
Male removed	0/11	2/8	2/19
Total	0/22	3/17	3/39

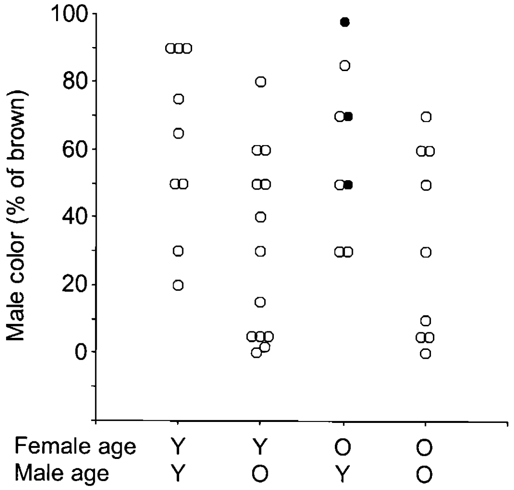

FIG. 1. Male coloration of female–male pairs of different age in the Pied Flycatcher. Y = yearling; O = older; filled circles = EPF nests.

these values are close to the expected value (D = 0.550).

Polygyny frequency.—Five of the males in this study were known to be polygynous and thus the dataset includes five primary nests and four secondary nests. The frequency of polygyny did not differ statistically with respect to breeding density, though it was twice as high in low density than in high density (high density: 9% (2/22); low density: 18% (3/17); Fisher's exact test, $P = 0.64$). No polygynous males were cuckolded. The difference in EPF rate between monogamous and polygynous males was not statistically significant (Fisher's exact test, $P = 1.00$).

Effects of breeding density and male removal on EPFs.—All three EPF nests were found in the low density areas (Table 1). In two of those cases, the male was removed. EPF rate was significantly associated with neither breeding density (Fisher's exact test, $P = 0.07$) nor male removal (Fisher's exact test, $P = 0.61$). Still, the difference in rate between densities was remarkable (0 vs. 18%), but the power of test was low. The chance of detecting that size of difference was only 37%. There was no difference in timing of nesting between pairs with and without EPY (Onset of egg laying: $\bar{x} \pm SD = 29.0 \pm 1.0$, $n = 3$ and 29.3 ± 2.1, $n = 36$; $t = 0.20$, $P = 0.84$).

Male and female characteristics and EPFs.—We used logistic regression analyses to study whether measured male and female traits had a significant effect on the probability of having EPY. We tested whether the introduction of each variable improved the model significantly. The probability of a female having EPY increased significantly with tail length ($b = 1.6$, $a = -85.0$, $n = 39$, $\chi^2 = 9.15$, df = 1, $P = 0.003$) and there was a slight increase with wing length ($b = 0.7$, $a = -55.6$, $n = 39$, $\chi^2 = 3.19$, df = 1, $P = 0.07$). Further, browner males had higher, but not statistically significant, probability to rear EPY than black males ($b = 0.05$, $a = -5.1$, $n = 39$, $\chi^2 = 3.35$, df = 1, $P = 0.07$; Fig. 1). Other traits did not contribute to the logistic regression models ($P > 0.10$ for all other traits).

Yearling males suffered more often from EPFs than older males (yearlings 3/17 and older males 0/22). Among females, the trend was in the opposite direction. The proportion of females engaged in EPFs was higher among older females (yearlings 0/22 and older females 3/17). However, those differences were not quite statistically significant (Fisher's exact test, $P = 0.07$ for both). In fact, all pairs that reared EPY consisted of an older female and a yearling male (Fig. 1).

Between population comparison.—There are data on EPF rate in Pied Flycatcher available from five earlier studies with variation in both breeding density and EPF rate (Table 2). This study provides two more data points to analyze the effect of breeding density on EPF rate. Gelter and Tegelström (1992) gave only range for the breeding density (1 to 2 pairs/ha), so here we used the value 1.5 pairs/ha in our analysis. However, the result did not change remarkably when either the lower or higher value was used. The brood EPF rate reported in those Pied Flycatcher studies was not correlated with breeding density ($r_s = -0.02$, $P = 0.97$, $n = 7$; Table 2, Fig. 2).

TABLE 2. Extrapair fertilization rate in the Pied Flycatcher according to different studies.

Site	EPF rate (%) Broods (n)	EPF rate (%) Nestlings (n)	Breeding density (pairs/ha)
Konnevesi, Finland[a]	0 (23)	0 (115)	1.8
Lingen/Emsland, Germany[b]	13 (31)	5 (165)	0.3
Oslo, Norway[c]	15 (18)	4 (98)	1.0
Konnevesi, Finland[d]	15 (20)	9 (110)	0.03
Oslo, Norway[e]	17 (27)	7 (135)	1.6
Konnevesi, Finland[f]	22 (36)	11 (223)	0.5
Uppsala, Sweden[g]	43 (7)	24 (38)	1–2

[a,d] This study, [b] Brün et al. 1996, [c] Lifjeld et al. 1991, [e] Ellegren et al. 1995, [f] Rätti et al. 1995, [g] Gelter and Tegelström 1992.

DISCUSSION

Effect of breeding density.—The influence of breeding density on EPF rate was apparent in previous studies of Red-winged Blackbird (*Agelaius phoeniceus*), Eastern Bluebird (*Sialis sialis*), House Finch (*Carpodacus mexicanus*), Great Reed Warbler (*Acrocephalus arundinaceus*), and Bearded Tit (*Panurus biarmicus*) (Gibbs et al. 1990, Gowaty and Bridges 1991b, Hill et al. 1994, Hasselquist et al. 1995, Hoi and Hoi-Leitner 1997) whereas there was no such effect in Bobolink (*Dolichonyx oryzivorus*), Tree Swallow (*Tachycineta bicolor*), Yellow Hammer (*Emberiza citrinella*), and Hooded Warbler (*Wilsonia citrina*) (Bollinger and Gavin 1991, Dunn et al. 1994, Sundberg and Dixon 1996, Tarof et al. 1998). Breeding density differences have been proposed as explainations for differences in EPF frequency found among the studies conducted in the Pied Flycatcher (Lifjeld et al. 1991, Gelter and Tegelström 1992) although that hypothesis has also been questioned (Rätti et al. 1995, Brün et al. 1996). In this study, we did not find any evidence for the increase of EPF rate with increasing breeding density as stated by density hypothesis (see Westneat et al. 1990, Birkhead and Møller 1992). The EPF rate was not correlated with breeding density between populations either. The density manipulation of the present study suggests rather that EPF rate was higher in low breeding density. The problem here is that the power of test was low due to small sample size.

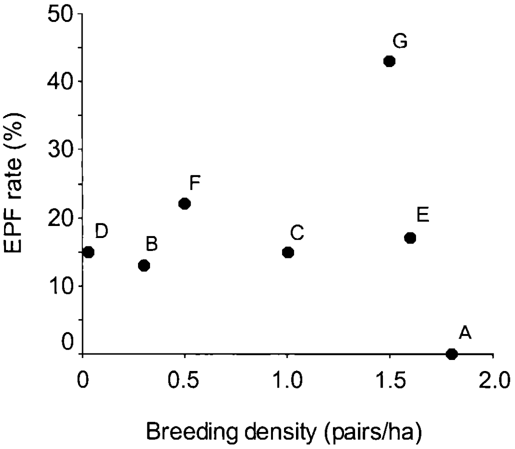

FIG. 2. Extrapair fertilization rate (proportion of broods with EPY) against breeding density in the Pied Flycatcher according to different studies. (See Table 2 for lettering designations.)

One possible factor that may influence EPF rate and cause differences among species is mating pattern. The Pied Flycatcher is a polyterritorial polygynous species, whereas previous studies have concerned monogamous or monoterritorially polygynous species. In the Pied Flycatcher, frequency of polygyny tends to be higher at low breeding density, at least if there is an excess of nest boxes (Alatalo and Lundberg 1984), which suggests that behavior of males depends partly on breeding density. Many males are polyterritorial, and it might be that at low breeding density males leave their first territories more often during the fertile period of the female. At high density, males' territories are often visited by intruders (Björklund and Westman 1983, Alatalo et al. 1987) and males in such territories may delay or give up the establishment of a second territory to be better able to guard their mate and territory. That might balance EPF rates between different densities. However, our male removal experiment suggests that absence from the territory may not increase EPF risk considerably.

Brün et al. (1996) found that polygynous male Pied Flycatchers were more likely to suffer from EPFs (but see Lifjeld et al. 1991) and suggested that the variation in EPF rate among populations could be explained by variation in polygyny rate. In the present study, however, all polygynous males sired all nestlings in both their nests. We cannot tell whether the cuckolded males were polyterritorial or not because the polyterritorial behaviour of those particular males was not studied.

Polyterritorial males are known to sometimes have their second territory or nest very far away, up to some kilometres, from their first nest (von Haartman 1956, Silverin 1980, Rätti et al. 1994). Moreover, in the low-nest-site density area, males readily travel longer distances to take over second territories (Rätti and Alatalo 1993). Thus, there hardly is any lack of potential extrapair mates for females even in low-breeding-density areas. All EPFs in our experiment occurred at the middle of the breeding season which is the time when the first breeding males are looking for their second territory.

Effect of male removal.—We did not find any significant influence of male removal on the occurrence of EPFs. The occurrence of EPFs was low also among males that were removed for 4 h. Earlier male removal studies have shown that during the female's fertile period, male removal increases the frequency of EPCs (Björklund and Westman 1983, Møller 1987, Birkhead et al. 1989, Björklund et al. 1992, Westneat 1994) and EPFs (Westneat 1994, Lifjeld et al. 1997b). There are, however, some possible explanations for the absence of an effect of male removal in our experiment. The duration of removal may have been too short or the timing of removal may have been wrong to induce any effect. However, in an earlier Pied Flycatcher study by Björklund and Westman (1983), the EPC risk increased even with shorter removal periods (1–2 hours). Also, our 4 h removal period might not have changed male presence significantly compared to the average male presence under natural conditions because polyterritorial males often leave their territories even before the start of egg laying (von Haartman 1956, Silverin 1980, Lifjeld et al. 1991, Rätti and Alatalo 1993).

Male and female characteristics.—There was some degree of association between EPFs and age dependent morphological traits (female tail and wing length, and male coloration). In all three cases of EPF found in present study, an older female was mated with a yearling male. That suggests that older females who mated with yearling males may have adjusted their initial mate choice through EPCs. Pied Flycatcher females thus may prefer older and darker males as extrapair mates, which contrasts with the study by Lifjeld et al. (1997a) which found that darker and older males more often rear EPY. The significance of plumage color in Pied Flycatcher mate choice has been controversial, because previous experimental studies have failed to find any female preference for male color (Alatalo et al. 1986, 1990), whereas there is some correlational evidence for such female preference (Järvi et al. 1987, Lifjeld and Slagsvold 1988b, Alatalo et al. 1990, Slagsvold and Dale 1994, Dale and Slagsvold 1996). A recent experimental study by Sætre et al. (1994) also provides evidence that females prefer darker and older males.

By preferring an older male as an extrapair mate, a female cannot increase the extent of paternal care because extrapair matings seldom, if ever, lead to extramale help in feeding nestlings (see Lifjeld and Slagsvold 1988a). In our investigation, we do not know which kind of extrapair mate that females had, although they were presumably darker than the current mate because cuckolded males were among the brownest. Another possibility is that younger and browner males are poor mate guarders compared to older ones. However, yearling males mated with yearling females did not suffer from EPFs, which suggests that yearling males are not poor in guarding abilities but that old females actively seek for EPFs.

Younger males have been observed to suffer more often from EPFs (Morton et al. 1990, Gowaty and Bridges 1991a, Weatherhead and Boag 1995) though many studies have failed to find any effect of male age (Hill et al. 1994, Rätti et al. 1995, Krokene et al. 1996, Sundberg and Dixon 1996). In two studies, older males reared EPYs more often (Westneat 1990, Lifjeld et al. 1997a). Most studies have not found an effect of female age (Morton et al. 1990, Gowaty and Bridges 1991a, Westneat 1992, Rätti et al. 1995, Barber et al. 1996, Sundberg and Dixon 1996). However, there is one study reporting a result similar to ours where old female Bobolinks and young males most often reared EPY in their nest (Bollinger and Gavin 1991).

In the Pied Flycatcher male, plumage coloration is correlated with age (Lundberg and Alatalo 1992). Thus, it is difficult to separate the effect of color and age. However, it may not be male age or quality per se, but the difference between characteristics of the current mate and the extrapair mate that affects female propensity to engage in EPCs (see Sundberg and Dixon 1996).

Concluding remarks.—We did not find any evidence that EPF rate would increase with breeding density as stated by the density hypothesis (Westneat et al. 1990, Birkhead and Møller 1992). Neither was there evidence for an effect of male removal during the female's fertile period. All EPF nests were reared by younger and browner males, and older long winged females, which suggests that females may adjust their mate choice after mating to acquire indirect genetic benefits by EPCs. That could also explain why the results suggest, contrary to expectations, that EPFs may be more frequent at low breeding density. Pied Flycatcher females have a restricted mate-search pattern (Dale et al. 1990, 1992; Slagsvold and Dale 1994, Hovi and Rätti 1994) and, thus, may be constrained to initially accept a mate of lower than preferred quality. Such constraints are likely to be more apparent at low than in high breeding density (Alatalo et al. 1988, Slagsvold and Lifjeld 1997). The sample size, and therefore the power of tests of this study was low for detecting statistical significe, even for quite noticeable and biologically meaningful differences between treatments (see Stoehr 1999). Therefore, more experimental studies are needed on this topic to test if the trends observed here are robust.

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