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## HIGH FREQUENCY OF EXTRA-PAIR PATERNITY IN EASTERN KINGBIRDS

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**Abstract.** Genetic parentage in the socially monogamous and territorial Eastern Kingbird (*Tyrannus tyrannus*) was examined in a central New York population by multilocus DNA fingerprinting. Extra-pair young were identified in 60% (12 of 20) of nests. Of the 64 nestlings profiled, 42% were sired by extra-pair males, but no cases of conspecific brood parasitism were detected. These results are markedly different from a previous electrophoretic study of the same species in a Michigan population, which reported 39% of nestlings were unrelated to one (typically the mother, quasiparasitism) or both (conspecific brood parasitism) of the putative parents. In the New York population, extra-pair paternity was most common among females that returned to breed on a former territory. Among females that were new to a breeding territory, extra-pair paternity increased directly with breeding density. Although the power of the tests was low, neither breeding synchrony nor male experience with a breeding territory appeared to be associated with the occurrence of extra-pair young.

**Key words:** *DNA fingerprinting, Eastern Kingbird, extra-pair fertilization, parentage, Tyrannus tyrannus.*

### Alta Frecuencia de Paternidad Extra-Pareja en *Tyrannus tyrannus*

**Resumen.** Se examinó la paternidad genética de *Tyrannus tyrannus*, especie socialmente monógama y territorial, mediante la técnica de huellas dactilares genéticas de múltiples loci en una población de Nueva

York central. En el 60% (12 de 20) de los nidos se identificaron juveniles con origen extra-pareja. De los 64 pichones investigados, el 42% fue engendrado por machos fuera de la pareja, aunque no se detectaron nidadas con parasitismo conespecífico. Estos resultados son considerablemente diferentes a los obtenidos en un estudio previo para la misma especie en una población de Michigan, el cual reportó que el 39% de los pichones no estaban relacionados con uno (típicamente a la madre, cuasiparasitismo) o ambos (parasitismo de nido conespecífico) padres putativos. En la población de Nueva York, la paternidad extra-pareja fue más común entre hembras que retornaron a criar a territorios que habían ocupado previamente. Entre las hembras que ocuparon por primera vez un territorio de cría, la paternidad extra-pareja aumentó directamente con la densidad de individuos reproductivos. A pesar que el poder del análisis fue bajo, ni la sincronía reproductiva, ni la experiencia de los machos en sus territorios de cría, parecen estar asociados a la ocurrencia de juveniles extra-pareja.

Genetic monogamy is now accepted as the exception rather than the rule among socially monogamous passerines that breed outside of the tropics. Westneat and Sherman (1997) showed, for instance, that extra-pair young (EPY) were found in 86% of passerine species for which data were available ( $n = 49$  species), and that on average, 18% of young were unrelated to one of the parents, usually the male. Although a finding of extra-pair paternity is no longer surprising, it is still unclear why extra-pair fertilizations (EPFs) occur so ubiquitously. Adaptive scenarios based on female control of EPFs include the possibility that females acquire good genes, produce a more genetically diverse brood, gain insurance against male infertility, or obtain material benefits from extra-pair males.

Questions also remain as to why the frequency of EPFs varies so widely among species. Two potentially

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important factors are breeding density (Westneat and Sherman 1997) and breeding synchrony (Stutchbury and Morton 1995). Frequent contact among individuals breeding at high density may increase the potential for EPFs (e.g., Reyer et al. 1997), and according to Stutchbury and Morton (1995), so should high breeding synchrony if females control and seek extra-pair copulations. In fact, Stutchbury and Morton (1995) proposed that the degree of overlap in female fertile periods was the primary contributor to interspecific differences in EPF frequency. They hypothesized that synchronously breeding species should have the highest EPF frequencies because females can simultaneously compare many males and better select extra-pair mates. Much of the available data support their hypothesis (e.g., Stutchbury et al. 1998, Chuang et al. 1999), but more data are needed to test the breeding synchrony hypothesis, and to test for an impact of other factors such as breeding density, parental age, and breeder experience on the occurrence of EPFs.

The Eastern Kingbird (*Tyrannus tyrannus*) is a socially monogamous passerine that has been reported to have a high frequency of extra-pair parentage. Curiously, females were usually excluded as the parent of the nearly 40% of young that were identified as extra-pair in a Michigan population (McKittrick 1990). As part of a larger study investigating the evolution of reproductive behavior in kingbirds, we used multilocus DNA fingerprinting to determine parentage and measure the frequency of lost parentage among both males and females in a New York population. We also attempted to determine if nest density, nesting synchrony, or parental experience with a territory were associated with extra-pair parentage.

## METHODS

Fieldwork was conducted in Delaware (42°78'N, 74°53'W) and Otsego (42°28'N, 75°03'W) Counties in central New York, where a color-banded population of Eastern Kingbirds has been under study since 1989 (Murphy 1996, 2000). Nests were located prior to egg laying by censusing all potential and former territories. We used adult behavior (territorial defense and feeding of nestlings) to identify putative parents at individual nests. Nearly half of the adults upon which our work was based were banded in a previous year, and none changed mates during the study. Mate replacement is a very rare phenomenon in this population (only one case in 11 years among marked birds); therefore we feel confident that all of the birds that were unbanded at the start of the study remained with their partners through the entire nest cycle.

Adults were captured using mist nets when nestlings were 11–14 days of age. Birds were weighed and measured, and blood samples (<100  $\mu$ L) taken via brachial venipuncture. Unmarked birds were banded with one aluminum U.S. Fish and Wildlife Service band and a unique combination of three color bands. Nestlings were weighed, measured, banded, and bled (50–100  $\mu$ L, brachial venipuncture) when 13 days of age. In all cases blood samples were collected in heparinized capillary tubes and immediately suspended in 1000  $\mu$ L of lysis buffer (Longmire et al. 1988). Blood samples collected in 1994 ( $n = 20$  families) were stored at room

temperature for several months, but unfortunately, the DNA from nearly all the nestling samples was severely degraded (adult DNA was fine; see also Conrad et al. 2000) and no usable samples resulted from the 1994 season. Thus, in 1995 and 1996 we kept blood on ice while we were in the field, and after transport to the laboratory, we stored all samples at 4°C until the DNA was isolated (2–3 days later). Usable fingerprint profiles were generated for 20 families, 41 adults, and 64 nestlings. The odd number of adults is attributed to a nest at which three adults were captured (two males and one female). All young hatched from clutches of 2 ( $n = 1$ ), 3 ( $n = 11$ ), or 4 ( $n = 8$ ) eggs, but three of the nests having four young yielded samples for only three nestlings because of either the loss of a nestling to predators or insufficient DNA.

Total genomic DNA was isolated as in Fleischer et al. (1994). Multilocus DNA fingerprinting was performed using the Jeffreys' 33.15 probe (Jeffreys et al. 1985) and an M13 bacteriophage DNA probe for a subset of seven families, four in which EPFs were and three in which EPFs were not detected (Vassart et al. 1987). Standard fingerprinting methods were followed (see Loew and Fleischer 1996 for protocols), with the exception of labeling M13 probe with [ $\gamma^{32}$ P] dATP instead of [ $\gamma^{32}$ P] dCTP, and then precipitating with salt, ethanol, and yeast tRNA. The DNA fingerprints for each family, arranged with nestlings flanked by their putative parents, were manually scored on acetate sheet overlays (Westneat 1990), and the number of novel bands and bands shared with one or both parents counted.

## STATISTICAL ANALYSES

We calculated band-sharing coefficients ( $S$  of Lynch 1990) between nestlings and putative parents to resolve parentage.  $S$  was calculated as twice the number of shared bands divided by the total number of bands for both individuals. The expected level of  $S$  for first-order relatives was estimated by assuming that an adult was the unambiguous parent of a nestling if there were no novel bands in the nestling's fingerprint (0.55; Table 1). The expected  $S$  for first-order relatives ( $R = 0.50$ ) was calculated from the background level of  $S$  and equation 22 of Lynch (1990) as 0.56, very close to the mean values of  $S$  for females and for unexcluded males with offspring (Table 1). To determine the lower limit of  $S$  below which first-order relatives would not be expected, we calculated the 95% and 99% confidence intervals surrounding the average  $S$  for first-order relatives (0.55). These resulted in conservative lower limits of 0.38 and 0.32, respectively (Burke and Bruford 1987).

We did not know the ages of the adults because none were banded as nestlings. Instead, we categorized adults as either "experienced" or "inexperienced" based on prior use of a specific territory. An experienced bird was a banded adult that bred on a territory in one year and returned to breed on that same territory in the next year. The designation of a bird as inexperienced meant that it was unbanded and new to a territory. These latter birds were known to be new to a territory because they replaced a banded bird that had bred on the territory in the previous season. An inexperienced bird may have bred elsewhere in the past,

TABLE 1. Band-sharing coefficients ( $S$ ) for presumptive father-offspring, presumptive mother-offspring, and unrelated adults (mated pairs) compared by the number of novel fragments. Results from the Jeffreys' 33.15 probe and M13 probe are summarized in (A) and (B), respectively. Values are mean  $S \pm SD$  ( $n$ ), and ranges.

Relationship	Number of novel fragments		
	0	0-2	$\geq 3$
(A) Jeffreys 33.15			
Presumptive father-offspring	$0.55 \pm 0.09$ (24)	$0.54 \pm 0.09$ (37)	$0.12 \pm 0.08$ (27)
Range	0.35-0.70	0.35-0.78	0.00-0.29
Presumptive mother-offspring	$0.54 \pm 0.10$ (24)	$0.54 \pm 0.10$ (37)	$0.56 \pm 0.10$ (27)
Range	0.37-0.69	0.33-0.76	0.40-0.70
Male-female pair	—	—	$0.12 \pm 0.09$ (26)
Range			0.00-0.30
(B) M13			
Presumptive father-offspring		0-1	$\geq 2$
Range		$0.48 \pm 0.14$ (13)	$0.16 \pm 0.12$ (7)
Presumptive mother-offspring		0.33-0.70	0.00-0.30
Range		$0.61 \pm 0.14$ (13)	$0.45 \pm 0.15$ (7)
Male-female pair		0.36-0.86	0.24-0.64
Range		—	$0.11 \pm 0.12$ (9)
			0.00-0.33

but we suspect that most inexperienced birds were probably first-time breeders because most kingbirds show high site fidelity between years (Murphy 1996).

We also evaluated the potential impact of breeding synchrony on extra-pair paternity (EPP) by calculating Kempnaers' (1993) breeding synchrony index for all females in the population. The index was calculated as the percentage of other females in the population whose fertile period overlapped that of the focal female, where the fertile period was assumed to extend from five days before the laying of the first egg until the laying of the penultimate egg of the clutch. We also calculated the local breeding synchrony index for all females for which we had fingerprints. The latter index was based on the fertile periods of the four females breeding closest to the focal female. We used the average distance to neighboring pairs as an index of breeding density, by measuring the shortest distance from a focal nest to nests of the same four pairs that were used to calculate local breeding synchrony. All nest locations were mapped on U.S. Geological Survey topographic maps (1:24 000) for another study before the fingerprinting results were obtained. We used SAS (SAS Institute 1990) and STATISTIX (Analytical Software 1994) to test for relationships between the presence of EPY and breeding experience, nesting synchrony, and density. Results are presented as means  $\pm$  SD. Tests are described in the Results, and unless otherwise stated, we assumed significance when  $P \leq 0.05$ .

## RESULTS

### DNA FINGERPRINTING RESULTS

The number of scorable bands in the 2-24 kbp range averaged  $14.0 \pm 3.0$  (range 8-21) for the Jeffreys' 33.15 probe. The proportion of nestlings with different numbers of novel bands was bimodal (24, 12, 1, 5, 4, 8, 8, and 2 with 0, 1, 2, 3, 4, 5, 6, and 7 novel bands, respectively), and the frequency of zero, one, or two novel fragments fit the Poisson distribution ( $\chi^2_1 = 1.0$ ,  $P > 0.5$ ). Based on the fit to the Poisson distribution,

we estimated mutation rate per individual ( $m$ ) to be 0.36 (Westneat 1990). The probability of a nestling having three bands due to mutation was thus  $4.7 \times 10^{-2}$  ( $0.36^3 = 3$  of 64 young), which suggested that three novel bands could be used as a cutoff to identify EPY. Thus, our final criteria for using the Jeffreys' 33.15 probe to identify EPY were that nestlings have three or more novel fragments and an  $S$  below 0.32 with one or both parents. The M13 probe revealed fewer fragments than the Jeffreys' 33.15, with an average of  $9.7 \pm 2.3$  bands scored per individual. The mutation rate,  $m$ , was 0.23, so we used a cutoff of two novel bands to identify EPY.

Based on Jeffreys' 33.15, EPY were detected in 12 of 20 nests (60%), and involved 27 of 64 nestlings (42%; Fig. 1). The mean  $S$  between presumptive female parents and nestlings did not vary with the number of novel bands (Table 1), and averaged  $0.55 \pm 0.10$  ( $n = 64$ ). Conspecific brood parasitism was thus never detected, and the probability of including a nonrelative as the female parent was  $9.9 \times 10^{-5}$  (Burke et al. 1989). On the other hand, mean  $S$  between the presumptive father and young varied with the number of novel bands, owing to the very low  $S$  of young with three or more novel bands (Table 1). The average  $S$  of the latter group of young with the presumptive father was identical to that of unrelated adults in the population (Table 1). The probability of inclusion of a non-father was  $2.1 \times 10^{-4}$  (Burke et al. 1989). EPP was thus common and accounted for all cases of lost parentage.

The probability of undetected instances of extra-pair fertilizations was relatively low for this sample of 64 nestlings ( $1.3 \times 10^{-2}$ ). There was, however, one case of ambiguous paternity. We captured two adult males and a female at a nest with three offspring in 1995. The two males were determined to be first-order relatives (possibly brothers) based on a high  $S$  (0.64). Neither male could therefore be excluded as the true parent of the young. The primary male (netted closer to

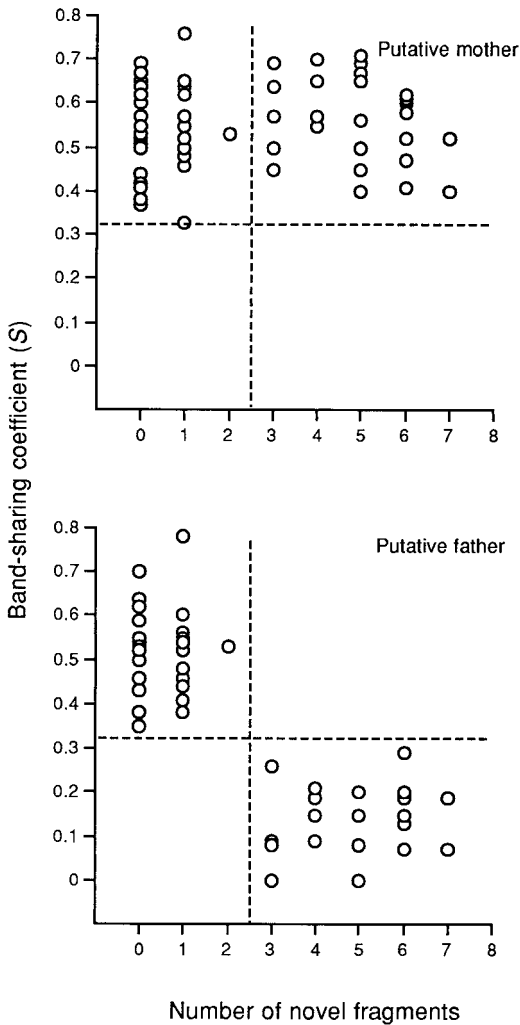


FIGURE 1. Examination of the occurrence of extra-pair young in Eastern Kingbird nests in New York. Number of novel fragments and band-sharing coefficients ( $S$ ) of young with the presumptive mother (upper frame) and presumptive father (lower frame) for the Jeffreys' 33.15 probe data only. The dashed lines indicate cutoff points for extra-pair young ( $\geq 3$  novel bands and  $S < 0.32$ ). Points in the lower right quadrant represent extra-pair young.

the nest and having slightly higher  $S$  with the offspring) was assumed to be the true father and no nestlings were considered EPY ( $S = 0.52, 0.54,$  and  $0.64$ ). The female parent and the apparent male parent from 1995 returned to breed on the same territory in 1996 (the only pair to contribute more than one fingerprint) and we again sampled the family unit. Surprisingly, in this pair's second year, the male did not father any of the young ( $S = 0.08, 0.09,$  and  $0.15$ ).

Seven broods, four of which had EPF young ac-

ording to the 33.15 probe, were reanalyzed with M13. The probability of assigning an unrelated male as father was  $5.2 \times 10^{-5}$ . Background band-sharing was also low ( $S = 0.11 \pm 0.12$ ), and we calculated the lower limit of the 95% and 99% confidence intervals for male-offspring relatives as 0.29 and 0.22, respectively. Exclusions were clearly supported for 7 nestlings excluded by the Jeffreys' 33.15 data ( $\geq 3$  novel bands,  $S < 0.29$ ; Table 1). For three nestlings, each with only a single novel M13 fragment, two had  $S$  of 0.46 and 0.40, while one had an  $S$  of 0.15. This third individual was not excluded based on the Jeffreys' 33.15 probe (1 novel fragment,  $S = 0.46$ ). The 10 remaining nestlings were not excluded based on M13 data (as we also concluded using the Jeffreys' 33.15 probe). Thus, results from the two probes differed slightly for only 1 of 20 nestlings, and we are confident of the exclusions made using the 33.15 data alone.

#### INDIVIDUAL AND ECOLOGICAL CORRELATES OF EPP

The distribution of EPY among nests appeared bimodal. Eight broods contained no EPY, two broods (brood size = 3 and 4) contained one EPY each, but 10 broods had at least half of the young fathered by a male other than the presumptive father (7 broods of 3 and 3 broods of 4). Three males in fact failed to father any of the young in their nest (all broods of three). Assuming all young had a probability of 0.42 of being EPY, we calculated the number of broods that would be expected to have EPY following methods described in Lifjeld et al. (1993). The predicted number of broods with EPY was 16.4 (out of 20), which differed marginally from the observed value of 12 ( $\chi^2_1 = 3.282, P = 0.07$ ; cell totals corrected for small sample size). In an attempt to explain this pattern, we tested four potential ecological correlates of extra-pair paternity (EPP): male and female breeding experience, breeding synchrony, and breeding density.

All six females that had experience on their territory in the past year had EPY, compared to only 6 of 14 inexperienced females ( $2 \times 2$  contingency table, Fisher's exact test,  $P = 0.04$ ). In contrast, there was no association of EPP with male experience (Fisher's exact test,  $P = 0.64$ ). In addition, we compared the number of nests with EPY between pairs that were above versus below the median breeding synchrony index. On both a population and local level, the occurrence of EPP was independent of breeding synchrony (Fisher's exact test,  $P = 0.65$  for both tests), but there was a tendency for the number of EPY to vary inversely with average nearest neighbor distance (calculated as the average distance to the four nearest neighbors). Eight of 10 nests below the median nearest neighbor distance held EPY compared to 4 of 10 above the median nearest neighbor distance (Fisher's exact test,  $P = 0.17$ ). A natural break in EPY frequency occurred at a nearest neighbor distance of 1 km: 10 of 13 pairs with an average nearest neighbor distance  $< 0.9$  km yielded EPY compared to 2 of 7 nests with an average nearest neighbor distance greater than 1 km (Fisher's exact test,  $P = 0.06$ ).

As a final test, we performed a Poisson regression to simultaneously examine the influence of female and male experience, breeding synchrony, and nearest



neighbor distance on the number of EPY in a nest. All four variables were entered, but then removed in a backward stepwise procedure until we were left with only those that significantly reduced the model's deviance. The model retained female experience ( $P = 0.02$ ) and average nearest neighbor distance ( $P = 0.02$ ). Separate comparisons of the number of EPY to nearest neighbor distance for experienced and inexperienced females showed that nests of experienced females held EPY regardless of density ( $b = -0.001$ ,  $P = 0.76$ ), whereas EPY were less likely to be found in the nests of inexperienced females as the average distance to neighbors increased ( $b = -0.10$ ,  $P = 0.01$ ). Least-squares linear regression indicated that 32% ( $P = 0.04$ ,  $n = 14$ ) of the variation in the number of EPY could be accounted for by nearest neighbor distance among the inexperienced females.

## DISCUSSION

Our results provide evidence of a very high frequency of extra-pair paternity (60% of nests and 42% of offspring) in an Eastern Kingbird population from central New York. Our recorded rate of EPP ranks among the highest yet reported for a socially monogamous passerine (Fleischer 1996, Westneat and Sherman 1997). As noted above, mate replacement is a very unlikely explanation for our results since over an 11-year period we have only once found that a banded male was replaced during the nesting cycle. Furthermore, we did not increase the frequency of EPP through our capture efforts because adults were not handled until nearly the end of the nestling period. We are thus confident that the adults identified as the putative parents were together from the start of nesting and that they behaved normally. In addition, stored sperm from copulations that might have occurred prior to pair formation is a very unlikely explanation for the high rate of EPP because egg laying generally occurs two, and often three, weeks after pairs form (but see Oring et al. 1992). Given the importance of last-sperm precedence (Birkhead and Møller 1992), copulation prior to pairing is probably of little consequence to kingbirds. We thus believe that the EPP that we documented is the result of normal extra-pair fertilizations.

McKittrick (1990) also found, using protein electrophoresis, a very high rate of extra-pair parentage in a Michigan population of kingbirds (39%), but oddly, the female was usually excluded as the probable parent. Her data suggested that the social mother lost parentage as a result of both quasiparasitism (an unrelated female breeds with the mate of a female and then lays in the latter's nest; Wrege and Emlen 1987) and conspecific brood parasitism. Quasiparasitism has only rarely been documented in other birds (Wrege and Emlen 1987, Birkhead et al. 1990, Alves and Bryant 1998), and conspecific brood parasitism (Rohwer and Freeman 1989) has repeatedly been shown to be much less common than EPP. Thus, our failure to exclude the social mother as the genetic mother of even a single nestling in the Charlotte Valley kingbird population leaves us with the difficult task of interpreting the contradictory results of McKittrick's (1990) study. One possibility is misidentification of the actual parents in the Michigan population. As described above, we have

found that a third bird occasionally attaches itself to a pair, generally late in the nest cycle. Although not common, it has occurred at least once in roughly every other year of our 12-year study. We assume that these birds failed in a nesting attempt elsewhere and redirected their parental care (Bragg 1968). McKittrick (1990) did not follow pairs throughout the nest cycle, and it is possible that some of the presumptive mothers that she collected were actually unpaired birds that associated with the nesting pair. Some of the difference might also be attributed to problems inherent to electrophoretic techniques of assessing parentage. For instance, differential gene expression between nestlings and adults may confound the assignment of parentage (Smyth et al. 1993), or the inherent low resolution of electrophoresis may make it difficult to discern whether observed mismatches are due to conspecific brood parasitism or EPFs (Romagnano et al. 1989, Smyth et al. 1993). It is also possible that the difference between the New York and Michigan populations is real, and although we regard it as unlikely, the possibility of such a major intraspecific difference warrants further study.

## CORRELATES OF EPP

Although many studies have shown that EPP is common among passerines, the individual and ecological predictors of EPP remain enigmatic. High nesting density, because it presumably increases the frequency of interactions between extra-pair individuals, has been argued to be an important contributor to the occurrence of EPP, but Westneat and Sherman's (1997) interspecific comparisons provide little support. Within species, high nesting density tends to be associated with frequent loss of paternity (Reyer et al. 1997, Westneat and Sherman 1997; but see Chuang et al. 1999). In our study, the number of EPY was negatively correlated with nearest neighbor distance among inexperienced females, suggesting an important influence of nesting density on the occurrence of extra-pair copulations. On the other hand, all experienced females obtained EPFs regardless of nearest neighbor distance.

The high frequency of EPP in kingbirds is consistent with Stutchbury and Morton's (1995) hypothesis that synchronously breeding species should exhibit the highest frequency of EPP. The synchrony indices for 1995 (0.55) and 1996 (0.54) rank among the highest recorded for temperate-zone breeding species (see Table 1 of Stutchbury and Morton 1995), and as their hypothesis predicts, the frequency of EPP in kingbirds is also high. On the other hand, we have no evidence that breeding synchrony affected the within-population probability of EPY, but we offer this only as a tentative conclusion because of the low power of our tests due to small sample size.

The strongest influence on the frequency of EPP appeared to be female experience: all of the females in our sample that returned to breed on a territory that they had used in the previous year obtained EPFs, regardless of their proximity to neighbors. Conversely, less than half of the inexperienced females had EPY in their broods. Most surprising was the fact that experienced males that were paired with a former mate (i.e., both partners bred on the same territory and with each other) frequently lost paternity, even of entire

broods. It is our view that experienced females, because they knew the locations of males in the surrounding landscape from the previous year, were able to obtain EPCs with little difficulty. The alternative interpretation of our data, that experienced females were more likely to suffer unwanted EPCs than inexperienced females, seems most unlikely. We therefore propose that experienced female kingbirds sought and obtained copulations from extra-pair males.

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