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Genetic evidence for multiple parentage in eastern kingbirds (*Tyrannus tyrannus*)

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Summary. Electrophoretic analysis was performed on 28 families of eastern kingbirds (*Tyrannus tyrannus*) from northern Michigan to estimate the occurrence of multiple parentage. Out of 19 families used in the final analysis, at least one putative parent was excluded in 9 families, or 18 out of 60 offspring (30% of offspring). Distribution of exclusion types conforms most closely to a model of quasi-parasitism, rather than extra-pair fertilizations, with secondary females laying their eggs in the primary female's nest, but "random" brood parasitism cannot be ruled out as an additional or alternative source of stray genes. Based on the model of random parasitism, an estimated 39% of all offspring in this population may be unrelated to one or both of the putative parents, or 53% based on a model of quasi-parasitism. Heretofore, eastern kingbirds have been considered to be exclusively monogamous; no behavioral evidence for alternative reproductive strategies has ever been reported for this species.

Introduction

The coupling of field and laboratory studies in recent years has indicated that extra-pair copulations (EPCs) may occur regularly in birds that are predominantly monogamous, as revealed by electrophoretic analysis (e.g., Westneat 1987b; Sherman and Morton 1988), and that intraspecific brood parasitism may provide an additional source of stray genes (e.g., Brown 1984; Wrege and Emlen 1987; Brown and Brown 1988). In the present study, I use allozymic variation to investigate the occurence of multiple parentage in kingbirds. The analysis was originally undertaken as a means of exploring a difference between heritability estimates based on male and female parents (see McKitrick 1986); in an earlier study of eastern kingbirds (McKitrick, unpublished), offspring appeared to resemble their putative mother more than their putative father in the size of a hindlimb muscle, M. flexor cruris lateralis. It appeared possible that differences in reproductive tactics between the sexes could lead to biases in the estimate of heritability depending on the sex of the putative parent. Published accounts (e.g., Bent 1942; Davis 1955; Murphy 1983b; Blancher and Robertson 1985) offer no evidence that extra-pair copulations or brood parasitism occur in eastern kingbirds, or that kingbirds are anything but exclusively monogamous.

Methods

Twenty-eight families of eastern kingbirds (*Tyrannus tyrannus*) including 49 adults and 85 offspring were collected in Emmet (14), Cheboygan (12), Alger (1), and Oscoda (1) counties in northern Michigan from June through August of 1985, 1986, and 1987 (22, 3, and 3 families, respectively). Parentage was determined by observation of adults feeding young in the nest and/or by nest defense behavior. No putative families were collected when more than two adults were in the vicinity of the nest, as sometimes happened when the researcher's presence caused a disturbance and if kingbird territories were unusually close together (e.g., if other kingbirds could be heard vocalizing in the area). Birds were collected within an hour after being located and identified as a family, or after a period of days if the nestlings were too small for accurate morphological measurement, or after a period of up to 2 weeks if the birds were under observation for another part of the study; no attempt was made to locate nests with eggs or to census nests to determine laying rates. An attempt was made to collect nestlings as close as possible to the time of fledging; the sample does contain younger nestlings, however (see Appendix 1). Approximate age was determined by comparing weight and/or developmental stage with that of nestlings of known age.

It was suspected that the adults in family # 2 might not be the parents of the single, fledged offspring in that group: they were not observed feeding the fledgling although they were hovering around it. This putative family was omitted from the analysis, although the adults were included in the calculation of allelic frequencies. All other families conformed to the criteria for parentage identification just described.

Whole specimens were maintained on wet ice in the field for up to 6 h. Fresh heart, liver, and muscle tissue from each specimen were preserved in liquid nitrogen and later transferred to a freezer at −70° C.

Tissue extracts for all specimens were subjected to horizontal starch gel electrophoresis using the methods described by Selander et al. (1971) and Harris and Hopkinson (1976). Details on the buffer systems are available from the author.

Hardy-Weinberg calculations were made using the BIOSYS-1 computer program (Swofford and Selander 1981). Probabilities of detection of extra-pair copulations (EPCs), intra-specific brood parasitism (ISBP), and quasi-brood parasitism (Q-ISBP) were calculated using the indices developed by Westneat et al. (1987). These analyses assume that mating is random with respect to genotype. Most polymorphic loci had >2 alleles, hence the three-allele equations were used to compute detection probabilities. These equations were included in a Lightspeed Pascal program for the Macintosh computer, supplied by D.F. Westneat (personal communication). For loci with >3 alleles, the rarest alleles were lumped (Westneat et al. 1987).

Despite the small sample size, I used the G-test (Sokal and Rohlf 1981) as a rough index of *goodness of fit* of observed to expected distributions of exclusions. In cases where frequency classes have <5 observations, it is recommended that some classes be lumped, and this was done in some cases by Westneat et al. (1987) and by Brown and Brown (1988). This is not entirely logical, however (and Westneat no longer advocates it; personal communication), as each of the classes is important in the analysis. I refrained from lumping classes and accepted the reduced power of the test that comes from using $f<5$. I also employed Williams' correction for a better approximation of x^2 and Yates' correction for observations of zero (Sokal and Rohlf 1981). This results in a conservative test *of fit*.

Results

Thirty-seven isozymes were scored for all 134 individuals. Seven of these presumptive genetic loci were sufficiently variable to be informative regarding the occurrence of multiple parentage: Est-1, Est-2, La, GDH, NP, Lgg, and ME-1. All seven loci were in Hardy-Weinberg equilibrium (Est-1 $x^2=0.03$, DF=1, $P=0.86$; Est-2 $x^2=4.10$, DF=10, $P=0.943$; Lal $x^2=0.95$, DF=3, $P=0.81$; GDH $x^2=3.60$, DF=1, $P=0.06$; NP $x^2=1.33$, DF=1, $P=0.25$; Lgg $x^2=0.03$, DF=1, $P=0.86$; ME-1 $x^2=0.51$, DF=6, $P=0.998$). Because electrophoretic resolution of the first sample (family # 1) was poor, this family was not considered further in this analysis. Family # 2 was also omitted for reasons explained in "Methods".

In 7 of the remaining families only one parent was available. Although one of these samples exhibited obvious exclusions, additional exclusions might have been detectable if the other parent had been available; therefore, in assessing the total number of exclusions, these samples were eliminated in order to avoid bias. In the remaining 19 families (60 offspring), there were 5 exclusions of the female, 1 of the male, and 15 ambiguous exclusions (either adult could have been the excluded one); there were no exclusions of both the male and female simultaneously. Genotypes for all individuals in the families showing exclusions are given in Appendix 1. The exclusions are distributed among 9 families, for a total of 47.4% of all families showing exclusions of one or more types. The total number of independent exclusions (21) is greater than the number of offspring excluded (18); different types of exclusions at different loci are considered to be independent, assuming the loci segregate independently (Westneat et al. 1987). In one family (# 6), all four nestlings showed exclusions at at least two loci, two families (# 4 and 26) had 3 out of 4 non-kin offspring, two (# 8 and 27) had 2 of 3 non-kin offspring, and four (# 3, 14, 21, 25) had 1 non-kin offspring. Brood size effects (Brown and Brown 1988) could not be calculated as nests were not always found at the same stage of the nesting cycle.

The probabilities of detecting non-kin offspring at each locus and the probability of detection over all loci are shown in Table 1 for the EPC and ISBP (intraspecific brood parasitism) models (Westneat et al. 1987). Table 2 shows the probability of detection over all loci for each type of exclusion, based on the probabilities at each locus from Appendix 2. The expected proportions of exclusion types (Table 2) are calculated by dividing the probability of detection of that type by the sum of the probabilities across types for each model (Westneat et al. 1987). The expected distributions were calculated by multiplying the expected proportions by the total number of observed exclusions (Table 3).

If extra-pair fertilization is the primay source of stray genes, male-only exclusions should outnumber female-only exclusions. The observed distribution (Table 3) clearly does not conform to this model ($G=21.64$, $df=3$, $P<0.001$; Sokal and Rohlf 1981). In this sample, maternal exclusions outnumber the paternal ones, which is consistent with a model of "quasi"-parasitism (Wrege and Emlen 1987); i.e., the parasite is a secondary female that mated with the attending male at that nest. In this case, the expected distribution of exclusion types should conform to the EPC model, but with the values for male-only and female-only exclusions reversed (Westneat et al. 1987; D.F. Westneat personal communication). This appears to be true in this sample ($G_{adj}=0.48$, $df=3$ $0.95<P<0.90$; Sokal and Rohlf 1981): the observed distribution is not significantly different from the expected. However, the model of "random brood parasitism" (ISBP) predicts that male

Table 1. Allele frequencies[a] and probability of detection for seven polymorphic loci in eastern kingbird tissue under the EPC (extra-pair copulation), ISBP (random intra-specific brood parasitism), and Q-ISBP (quasi-parasitism) models

Enzyme	Allele frequency	N	Probability of detection (d_i) (EPC or Q-ISBP)	Probability of detection (d_i) (ISBP)
Est-1	A 0.97 B 0.02 C 0.01	47	0.02939	0.05701
Est-2	A 0.65 B 0.06 C 0.03 D 0.23 E 0.02	47	0.26599	0.39930
La	A 0.86 B 0.09 C 0.05	47	0.12720	0.22358
GDH	A 0.94 B 0.01 C 0.05	47	0.05593	0.10443
NP	A 0.79 B 0.21	47	0.13838	0.22158
Lgg	A 0.93 B 0.07	47	0.06086	0.11067
ME-1	A 0.91 B 0.09	47	0.07519	0.13356
Overall probability of detection: $\left[=1-\prod_{i=1}^{n}(1-d_i)\right]$			0.560	0.764

[a] Based on the adults in all families except family # 1

Table 2. Probability of detection for each kind of exclusion. Numbers are based on data in Table 1 and Appendix 2

Type of exclusion:	Both male and female	Male only	Female only	Ambiguous
EPC model				
Probability of detection	0.000	0.227	0.000	0.412
Expected proportion of types	0.000	0.355	0.000	0.645
ISBP model				
Probability of detection	0.074	0.151	0.151	0.603
Expected proportion of types	0.076	0.154	0.154	0.616
Q-ISBP model				
Probability of detection	0.000	0.000	0.227	0.412
Expected proportion of types	0.000	0.000	0.355	0.645

Table 3. Observed and expected distributions of exclusions under the EPC, ISBP, and Q-ISBP models. Expected distributions are based on data in Table 2

Type of exclusion:	Both male and female	Male only	Female only	Ambiguous
Observed distribution Total: 21	0	1	5	15
Expected distribution: EPC model	0	7.5	0	13.5
Expected distribution: ISBP model	1.6	3.23	3.23	12.94
Expected distribution: Q-ISBP model	0	0	7.5	13.5

and female exclusions should be equal because nestlings from parasitism events should be unrelated to both attending adults (Table 3). This is not the case, but this model cannot be ruled out ($G_{adj}=5.17$, $df=3$, $0.3>P>0.2$).

Under the model of quasi-parasitism, the overall probability of detecting non-kin offspring is 0.560 (Table 1); therefore, the observed number of offspring exhibiting exclusions (18, or 30%; SD = 0.06; Westneat et al. 1987) is estimated to be 56% of the total non-kin offspring, or 32 offspring (53%) unrelated to at least one of the attending adults in this sample of 60 offspring. If mismatches were all due to "random" ISBP, the probability of detection would be 0.764 (Table 1), with an estimated 24 offspring (39%) out of the 60 being unrelated to one or both parents. A combination of these situations may obtain in eastern kingbirds.

Discussion

In the present study, allozymic variation was sufficient to reveal that a minimum of 18 out of 60 (30%) kingbird offspring were unrelated to at least one of the attending adults. Four families showed a maternal exclusion, indicating that intraspecific parasitism affected at least 4 out of 19 nests. If the 5 additional families with paternal or ambiguous exclusions are all due to brood parasitism as well, then a total of at least 9 out of 19 families were subject to parasitism (47%). The allozymic data do not support the occurrence of extra-pair copulations, although they may nevertheless occur. The distribution of exclusions (the number of female-only exceeds the number of male-only exclusions 5 to 1) suggests a model of quasi-parasitism, however, with secondary females laying one or more eggs in the primary female's nest. A larger number of male-only exclusions would be expected if extra-pair copulations were prevalent, or if the

parasitic females laid their eggs randomly rather than in the nest of their mate and his primary female (see Table 3). The fact that one male exclusion occurred suggests that random parasitism does occur as well. More accurate methods of determining the source of stray genes, such as DNA fingerprinting (Quinn et al. 1987; Wetton et al. 1987; Burke and Bruford 1987), will be necessary to confirm the occurrence and extent of both kinds of parasitism in eastern kingbirds.

Intraspecific brood parasitism has been reported in numerous nonpasserine birds (particularly anseriforms), but in relatively few passerine species (see review in Yom-Tov 1980). Its occurrence has been confirmed by behavioral observation in white-fronted bee-eaters (*Merops bullockoides;* Emlen and Wrege 1986), cliff swallows (*Hirundo pyrrhonota;* Brown 1984) and barn swallows (*Hirundo rustica;* Møller 1987), and by genetic analysis in white-fronted bee-eaters (Wrege and Emlen 1987), starlings (*Sturnus vulgaris;* Lombardo et al. 1989), cliff swallows (Brown and Brown 1988) and eastern bluebirds (*Sialia sialis;* Gowaty and Karlin 1984). Other instances in passerines have been inferred, e.g., by the appearance of more than one egg in the nest within a 24-h period (see references in Yom-Tov 1980 and Gowaty and Karlin 1984). Other genetic and behavioral studies have indicated that extra-pair copulation rather than brood parasitism is the most likely source of stray genes in indigo buntings (*Passerina cyanea;* Westneat 1987a, 1987b; Westneat et al. 1987) and mountain white-crowned sparrows (*Zonotrichia leucophrys oriantha;* Sherman and Morton 1988). Investigation of multiple parentage in other (both passerine and nonpasserine) species has met with variable success, depending on detectable allozymic variation (Gavin and Bollinger 1985, bobolinks; Joste et al. 1985, woodpeckers; Mumme et al. 1985, woodpeckers; Evarts and Williams 1987, mallards). The phenomenon has not been reported previously for any flycatcher (Tyrannidae), nor for any other suboscine passerine.

Eastern kingbirds generally lay between 2 and 4 eggs (Davis 1941, 1955; Morehouse and Brewer 1968; Murphy 1983a, 1983b, 1986a, 1986c); in an eastern Ontario population clutch size ranged from 2 to 5 (Blancher and Robertson 1985). In the present study, no nest contained more than 4 nestlings (I did not see many of the nests at the egg stage), so there was no *a priori* reason to expect brood parasitism. The genetic evidence for the phenomenon in kingbirds was unanticipated, as kingbirds are highly aggressive and defend their territories against intruders of either sex as well as against members of other avian species (Bent 1942; Davis 1941). Furthermore, kingbirds are "egg rejecters" (Rothstein 1975; Murphy 1986b) and tend to expel the eggs of heterospecific brood parasites such as brown-headed cowbirds (*Molothrus ater*); cowbird eggs are usually recognizably different from those of kingbirds. Females also reject their own eggs if these are altered by the addition of black ink; however, they will tolerate eggs marked with mercurochrome, which is closer than black ink in color to the natural reddish-brown spots of kingbird eggs (personal observation). Females therefore would probably be unlikely to reject other kingbird eggs, unless perhaps these were significantly different in appearance (e.g., size) from their own.

Brown (1984) observed brood parasitism among cliff swallows to occur within 60 s of the parasite entering the host's nest, and in one case the parasite deposited her egg in 15 s "while the nest owner was present but fighting another intruding conspecific in the nest entrance." If kingbirds are capable of such rapid egglaying as well, this could explain how females might successfully parasitize aggressive conspecifics.

The estimate of non-kin offspring in this kingbird population, based on a model of quasi-parasitism (53%), is higher than that reported by Brown and Brown (1988) for cliff swallows for a model of strict (random) brood parasitism (23.7%). The former model uses the same probability estimate as the EPC model. The probability of detecting strict brood parasitism in this kingbird population was 0.764 (Table 1), which would lead to an estimated 39% non-kin offspring. Romagnano et al. (1989) outlined numerous potential problems with electrophoretic studies of this kind. Several of these problems are built into this study because it started out as a heritability analysis, with tissues being saved because it was convenient to do so. For example, nests were not checked early in the season, so eggs were not counted daily nor were nestlings matched up with eggs. Furthermore, if the study had begun as a parentage analysis, possibly more time would have been spent observing adult behavior at the nests to ascertain parental identities; however, confidence of parentage is equally critical for heritability analyses and considerable attention was paid to this in the field. Of all the problems discussed by Romagnano et al., however, the one most likely to affect the present study is the small sample of families. This problem could be overcome in future studies of kingbirds only if the families were sampled non-destructively, as collecting was severely limited by the location

of the nests with respect to human dwellings. The low sample would be unlikely to affect the overall probability of detecting exclusions, but it could affect the observed distribution of exclusion types and hence conclusions about the prominence of any one reproductive strategy; it would certainly affect the power of the G-test. The minimum number of non-kin offspring found in this study is considerably higher, however, than that reported by Westneat et al. (1987), Wrege and Emlen (1987), Sherman and Morton (1988), or Brown and Brown (1988): 18 out of 60 nestling kingbirds (0.30) compared with 27 out of 160 indigo buntings (0.17), 7 out of 97 white-fronted bee-eaters (0.07), 15 of 110 white-crowned sparrows (0.14), and 35 out of 349 cliff swallows (0.10). Furthermore, as Westneat et al. (1987) point out, with probabilities of detection as high as those reported here (0.560 for EPC model, 0.764 for ISBP model), the electrophoretic data can be highly informative despite the small sample.

The increasing evidence for alternative reproductive strategies in apparently monogamous avian species has distinct sociobiological implications (see, e.g., Mock 1983; Sherman and Morton 1988). Of considerable interest, too, is its implications for studies of avian quantitative genetics: interpretation of heritability estimates may be compromised when parentage is uncertain. This theoretical problem was raised by Boag (1983) and by Alatalo et al. (1984), but to date no molecular genetic data have been available for use in conjunction with quantitative genetic data. This remains an important area for investigation.

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Appendix 1. Genotypes from all families for the Est-1, Est-2, La, GDH, NP, Lgg, and ME-1 loci, respectively. Genotypes indicating parental exclusions are shown in boldface

Field #	Family #	Sex	Age	Genotypes	Type of exclusion[a]	Approximate age of offspring (in days)
016	03	F	A	AA AA AA AA BA AA AC		14
017	03	M	A	AA AD BA AA AA AA AC		
018	03		J	AA DD AA AA BA AA AC	F	
019	03		J	AA AD AA AA BA AA AA		
020	03		J	AA AA AA AA AA AA AA		
021	03		J	AA AD AA AA BA AA AA		
022	04	M	A	AA AA AA AA AA AA AC		6
023	04	F	A	AA AD AA AA AA AA AA		
024	04		J	AA AA AA AA AA AA AA		
025	04		J	AA AD AA AA BA AA AA	A	
026	04		J	AA AD AA AA BA AA AA	A	
027	04		J	AA AD AA AA BA AA AC	A	
032	06	F	A	AA AA AA AA AA AA AA		14–15
033	06	M	A	AA AD AA AA AA AA AA		
034	06		J	AA BD BA AA BA AC AA	F, A, A, A	
035	06		J	AA AA BA AA BA AA AA	A, A	
036	06		J	AA AD AA AA BA AC AA	A, A	
037	06		J	AA BD AA AA AA AC AA	F, A	
043	08	F	A	AA AA AC AA BA AA AC		13
044	08	M	A	BA AA AA AA AA AA AA		
045	08		J	AA AA BA AA AA AA AC	A	
046	08		J	AA AA AC AA BA AA AA		
047	08		J	AA AD AA AA AA AA AA	A	
071	14	M	A	AA AE AA AC AA AA AC		14–15
072	14	F	A	AA AD BA AA BA AA AA		
073	14		J	AA AE BA AA BA AA AC		
074	14		J	BA AD AA AC BA AA AA	A	
075	14		J	AA AA BA AA AA AA AC		
076	14		J	AA AD AA AA AA AA AA		
088	18	M	A	AC AA AA AA AA AA AA		15
089	18		J	AA AD AA AA AA AA AA		
090	18		J	AA CD AA AA AA AA AA		
091	18		J	AA CD AA AA AA AA AA		
092	18		J	AA AD AA AA AA AA AA		
114	21	F	A	AA AD AC BA BA AA AC		14
115	21	M	A	AA AD AA AA BA BA AA		
116	21		J	AA DD AA BA AA BB AC	F	

Appendix 1. (continued)

Field #	Family #	Sex	Age	Genotypes	Type of exclusion[a]	Approximate age of offspring (in days)
117	21		J	AA AD AA AA BA BA AA		
118	21		J	AA DD AA AA AA AA AC		
169	25	F	A	AA DD AA AA AA AA AA		9
170	25	M	A	AA AA AC AA AA AA AA		
171	25		J	AA AA AA AA AA AA AA	F	
206	26	F	A	AA AD BA AA BA AA AA		8–9
207	26	M	A	AA BD AA AA AA BA AA		
208	26		J	AA BA BA AA BA AC AA	A	
209	26		J	AA BD AA AA BA BA AA		
210	26		J	AA DD BA AC AA AC AA	A, A	
211	26		J	AA AD BA AA AA BA AC	A	
219	27	F	A	AA AA AC AA BA AA AA		9
220	27	M	A	AA DD AA AA BB AA AA		
222	27		J	AA AA BA AA BB AA AC	M, A, A	
223	27		J	AA AD BA AA BB AA AA	A	
224	27		J	AA AD AA AA BA AA AA		

Total 18 individuals showing 21 independent exclusions out of 60 offspring in 19 families
[a] F = female only, M = male only, A = ambiguous

Appendix 2. Probability of detection for each type of exclusion (Westneat et al. 1987) at 7 protein loci under the EPC and ISBP models

Allele	Model	Type of exclusion			
		Both male and female	Male only	Female only	Ambiguous
Est-1	EPC	0.00000	0.00172	0.00000	0.02768
	ISBP	0.00082	0.00090	0.00090	0.05440
Est-2	EPC	0.00000	0.13015	0.00000	0.13581
	ISBP	0.03224	0.08656	0.08656	0.19395
La	EPC	0.00000	0.03120	0.00000	0.09600
	ISBP	0.01209	0.01840	0.01840	0.17468
GDH	EPC	0.00000	0.00648	0.00000	0.04946
	ISBP	0.00280	0.00355	0.00355	0.09452
NSP	EPC	0.00000	0.05505	0.00000	0.08333
	ISBP	0.01839	0.03665	0.03665	0.12988
Lgg	EPC	0.00000	0.00848	0.00000	0.05239
	ISBP	0.00369	0.00479	0.00479	0.09740
ME-1	EPC	0.00000	0.01342	0.00000	0.06178
	ISBP	0.00561	0.00781	0.00781	0.11234