POLYGYNY AND EXTRA-PAIR PATERNITY IN A POPULATION OF SOUTHWESTERN WILLOW FLYCATCHERS

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Abstract. Although polygyny can potentially increase male reproductive success, the benefits of this strategy could be offset by losses to extra-pair paternity or reduced offspring survival. We developed microsatellite markers to assess the influence of extra-pair offspring (EPO) on reproductive success and paternity in monogamous and polygynous pairs of the facultatively polygynous Southwestern Willow Flycatcher (*Empidonax traillii extimus*). Based on genotypes of 140 offspring from 56 clutches over six years, 14% of nestlings in our study population were extra-pair offspring, with 23% of all successful nests containing at least one EPO. We found that polygynous males produced 2.11 ± 0.35 offspring per season, compared to 1.15 ± 0.18 for monogamous males. This increased reproductive success was due primarily to the increased number of nests of polygynous males, as the number of offspring per pair did not differ between monogamous and polygynous males. Twenty of the 140 genotyped nestlings were extra-pair offspring. Sires could be assigned to 16 of these; one polygynous male sired two EPO in one nest, two monogamous males sired eight EPO in two nests, and four nonterritorial males sired six EPO in four nests. Overall, these results indicate that in this population, females of polygynous males did not raise a disproportionate number of EPO as a result of the polygynous mating strategy of their mate, and that both territorial and nonterritorial males sired EPO.

Key words: extra-pair offspring, microsatellite, polygyny, reproductive success, Willow Flycatcher.

Poliginia y Paternidad Extra Pareja en una Población de *Empidonax traillii extimus*

Resumen. A pesar de que la poliginia puede potencialmente aumentar el éxito reproductivo de los machos, el beneficio de esta estrategia puede ser contrarrestado por pérdidas ocasionadas por la paternidad extra pareja o por la reducción de la supervivencia de la progenie. Desarrollamos marcadores microsatelitales para determinar la influencia de la progenie extra pareja (PEP) sobre el éxito reproductivo y la paternidad en parejas monógamas y políginas de la especie facultativamente políginia *Empidonax traillii extimus*. Con base en genotipos de 140 crías provenientes de 56 nidadas en seis años, determinamos que el 14% de los polluelos en nuestra población de estudio fueron PEP y que el 23% de todos los nidos exitosos contuvo por lo menos una PEP. Encontramos que los machos políginos produjeron 2.11 ± 0.35 crías por estación, en comparación con 1.15 ± 0.18 crías producidas por machos monógamos. Este éxito reproductivo mayor se debió principalmente al mayor número de nidos de los machos políginos, ya que el número de crías por pareja no difirió entre machos monógamos y políginos. De los 140 polluelos analizados genotípicamente, 20 fueron PEP. Los padres progenitores pudieron ser identificados para 16 de estos polluelos. Un macho polígino engendró dos PEP en un nido, dos machos monógamos engendraron ocho PEP en dos nidos y cuatro machos no territoriales engendraron seis PEP en cuatro nidos. En general, estos resultados indican que en esta población las hembras de machos políginos no criaron un número desproporcionado de PEP como resultado de la estrategia de apareamiento polígina de sus parejas y que tanto los machos territoriales como los no territoriales engendraron PEP.

INTRODUCTION

In facultatively polygynous birds, males potentially increase their reproductive success by forming pair bonds with more than one female (social polygyny), by participating in extra-pair fertilizations (EPFs), or both. However, social polygyny and the pursuit of EPFs may involve a trade-off, with the resident male unable to spend as much time guarding his mate(s) from intruding males pursuing EPFs (Westneat et al. 1990, Westneat 1993, Hasselquist and Bensch

1991). Alternatively, if EPFs are driven by female choice (Birkhead and Møller 1993, Gowaty 1994, Stutchbury and Neudorf 1997), females may perceive polygynous males as higher quality mates and therefore be less likely to solicit fertilizations from monogamous males. As a result, polygynous males would be expected to have fewer extra-pair offspring (EPO) in their nests (Westneat et al. 1990, Møller 1992, Hasselquist 1994, Hasselquist et al. 1995, Hasselquist and Sherman 2001). Several field studies have documented more EPO in the nests of socially polygynous males compared to monogamous males in the same population (Gibbs et al. 1990, Dunn and Robertson 1993, Freeland et al. 1995, Soukup and Thompson 1997, Pilastro et al. 2002, Hamao and Saito 2005), while others have found no difference in EPO among polygynous and monogamous males (Lifjield et al. 1991, Westneat 1993). In light of these contrasting findings, it is difficult to predict what the relative reproductive success for polygynous and monogamous strategies will be without assessing genetic parentage.

Willow Flycatchers (*Empidonax traillii*) are facultatively polygynous, with 10%–50% of male Willow Flycatchers in a population having more than one reproductive female nesting within their territory (Sedgwick 2000, Davidson and Allison 2003; MJW and K. Enos, Southern Sierra Research Station, unpubl. data; R. McKernan and G. Braden, San Bernadino County Museum, unpubl. data). Nest success of polygynous and monogamous males did not differ in two Arizona populations, therefore polygynous males apparently realized higher reproductive success by having a greater number of nests (Davidson and Allison 2003). Male Willow Flycatchers do not participate in nest building, do not incubate eggs or feed incubating females, and spend little time feeding nestlings (Ettinger and King 1980). As a result, they can potentially spend time in pursuit of other mating opportunities. Although extra-pair copulations have been observed in free-living populations (E. Paxton, USGS Colorado Plateau Research Station, Northern Arizona University, unpubl. data; MJW, unpubl. data), neither the prevalence of extra-pair offspring, nor the comparative susceptibility of polygynous and monogamous pairs to extra-pair fertilizations, have been determined.

To investigate the predominance of extra-pair offspring in a population of the endangered Southwestern Willow Flycatcher (*Empidonax traillii extimus*), we developed eight microsatellite markers that allowed us to confirm parentage and detect EPO, thus providing a quantitative assessment of individual reproductive success. Specifically, we compared the reproductive success of monogamous and polygynous males to assess the influence of EPO on individual reproductive success.

METHODS

STUDY SPECIES

The Southwestern Willow Flycatcher population in the Kern River Valley in California was one of the largest known breeding populations of this subspecies in the mid- to late-1990s (Marshall 2000). From 1989 to 1998, this population consisted of approximately 27–39 breeding pairs, but has since declined to as few as 11 pairs (Whitfield et al. 1999, Kus and Whitfield 2005). Breeding behavior in the South Fork Kern River Valley population has been extensively monitored in the past twelve years (MJW and K. Enos, unpubl. data; MJW, unpubl. data), and over 80% of the population has been marked with both U.S. Fish and Wildlife Service aluminum bands and unique combinations of plastic color bands.

Willow Flycatcher females lay up to four eggs per nesting attempt, and may nest more than once per season. Mayfield nest success for this population of Willow Flycatchers has ranged from 16% to 61% (MJW and K. Enos, unpubl. data). Males of this population typically arrive on the breeding grounds between 10 May and 10 June and immediately begin to establish and defend a territory. Females arrive later (between 25 May and 20 June) and normally begin building nests within five days. It is not unusual for females to abandon their nests at any point during the typical seven days of construction. This, coupled with the likelihood of later abandonment due to egg and nestling predation or Brown-headed Cowbird parasitism, results in highly asynchronous female nesting and breeding cycles (MJW, unpubl. data). Males usually defend a territory that contains one or more females and are presumed to mate with all females in their territory and, occasionally, with females in other territories.

FIELD PROCEDURES

We used playback recordings of a singing male to survey for Southwestern Willow Flycatchers breeding in the study area (Tibbetts et al. 1994). When a flycatcher was detected by sight or sound, we recorded its location, identity (if banded), and breeding condition. Sex of breeding birds was determined based on behavior, e.g., singing by males and nest-building by females (Sedgwick 2000; MJW, pers. obs.). To ensure that our resident population estimates were not inflated by migrating birds, we surveyed each area three times between late May and early July. In addition, we revisited the locations of all sightings at least three times over a two-week period to confirm that the birds were breeding in the area and not simply passing through. Territories were visited at least four times per week from mid-May to August for purposes of banding, resighting, nest searching, and nest monitoring. Between 1995 and 2002 we found 225 nests, four of which were found empty, indicating either that the young had fledged or the nest had been depredated before we discovered it. Renesting attempts by three of the four pairs were observed, thus we are confident that we detected all, or nearly all, of the breeding birds within our study area. We banded adults with a unique combination of aluminum U.S. Fish and Wildlife Service and plastic color leg bands and searched territories for nests. When a nest was located, we checked it daily during the egg-laying stage and every two or three days during the incubation and nestling stages. For each breeding attempt we gathered data on the number of eggs laid, number of eggs hatched, number of fledglings, parasitism status, and causes of nest failure (if any).

Blood samples were obtained from most adults and nestlings from 1997 through 2002 by clipping a toenail above the quick (Busch et al. 2000). At the study site, samples were stored on ice in lysis buffer for up to 6 hours (10 mM Tris [Tris(Hydroxymethyl) Aminomethane], 1 mM EDTA [ethylenediaminetetraacetic acid], 1% SDS [sodium dodecyl sulfate], 100 mM NaCl, pH 8), then frozen at 0°C before being shipped to Northern Arizona University, where they were stored at −80°C in the lab for later DNA extraction and analysis.

DNA was extracted from blood samples using the procedure described by Müllenbach et al. (1989). DNA was resuspended in 50 µl of TE buffer [10 mM Tris, 1 mM EDTA]. Serial dilutions of each sample were made in water for final DNA concentrations of 10, 1, and 0.1 ng/µl. Samples were stored at −20°C.

Microsatellite markers were identified using an enrichment protocol (Li et al. 1997). Microsatellite repeats in the genome were enriched by selective hybridization to biotinylated oligonucleotide probes consisting of $(ACG)_5$, $(AAAG)_6$, $(CTT)_5$, and $(GATA)_6$ repeat motifs. Genomic microsatellites were recovered by linking the biotinylated probe to the streptavidin-coated surface of magnetic beads (Dynabead® M280 by Dynal, Carlsbad, California). A magnet was used to separate the hybridized microsatellites from unwanted genomic DNA. The magnetic beads were then removed. The microsatellites were inserted into pGEM plasmid vectors (Promega, Madison, Wisconsin), transformed into *E. coli* to create clone libraries, screened for size differences using PCR, then sequenced to characterize flanking sequences and to create microsatellite probes. Of the 82 clones that were sequenced, primers were designed for 29 that contained both microsatellite repeats and suitable flanking sequences for primer designation. Ten of the resulting primer pairs yielded polymorphic amplicons across individuals (Table 1). Approximate allelic size ranges were determined and the forward primers were each labeled with one of three different bioluminescent dyes such that all PCRs for each individual could be pooled and run on one lane of an acrylamide gel using an ABI 377 (Applied Biosystems, Foster City, California) or capillary-based automated sequencer. Fragment sizes were determined using GeneScan software (Applied Biosystems) and allele bin sizes were designated according to the size of the repeat motif using Genotyper Software (Applied Biosystems). Two of the ten markers did not amplify consistently during PCR and thus were not used for analysis.

PCR was performed in 10 µl reactions with final concentrations of 1× PCR Buffer (Gibco BRL, Invitrogen, Carlsbad, California), 3 mM $MgCl_2$, 0.2 mM dNTPs (deoxynucleotide triphosphates), 10 pM primers, 0.5 U Taq (*Thermus aquaticus*) DNA polymerase, and 0.2 ng template DNA. The complete thermal profile (94°C for 20 sec; 15 sec annealing at 65°C; and

TABLE 1. PCR primer loci, sequences, repeat sequences, size of cloned sequence, observed (H[O]) and expected (H[E]) heterozygosity levels, and number of alleles found in Willow Flycatchers (WIFL).

Locus (dye label)[a]	Primer sequences F: forward; R: reverse	Repeat sequences	Size (bp)	H(O)	H(E)	WIFL
GATA2 (FAM)	F:aatgagagtgataattatctgtgag R:aagcctaagatttcccactacttgacattttccttc	$(GATA)_{10}(TG)_3(TA)_2$	253	0.854	0.845	10
GATA5 (HEX)	F:aggcttattttcaagacaagcaaatggaaacat R:gaaggattcctttggtcttcaattattcacctatctt	$(GATA)_7GACA(GATA)_2$	202	0.772	0.814	9
GATA6 (FAM)	F:tgcccaaaattcctcaga R:atcaaagcagcatagtc	$(GATA)_{12}$	424	0.714	0.876	16
GATA7 (HEX)	F:ctccctcgagctggattgttattact R:ctaaaattccctcgcaaaacaacctcttctta	$(CTAT)_{16}CATCAT(CTAT)_2$	434	0.773	0.826	14
GATA9 (FAM)	F:gaggcaggtctcttattct R:tccgagcaagtttttccctggatttgagtgtg	$(CTAT)_4CTAC(CTAT)_{12}$	353	0.843	0.840	17
ACG1 (FAM)	F:cgcataaaacaaccaaaccct R:gctcagctccgaatttctca	$(GCT)_{16}$	160	0.251	0.260	8
ACG2 (NED)	F:cattacaagtgccatgtgct R:ctgcttgccttctctacgtg	$(AGC)_6$	175	0.525	0.472	4
ACG5 (HEX)	F:tggcgatggggaggcaccagagc R:gtccgtgagcagaagcctaaaacacagagt	$(GCT)_4GGT(GCT)_3CCT(GCCT)_3$	128	0.461	0.444	4
GATA1 (FAM)	F:attttgtaagtgccagagaattaccagaatattactatgctc R:actttgcctggaaaaacagagattttaaccatc	$(CTAT)_{18}$	319			
GATA3 (NED)	F:gactgaagttgccaaggcaaaatgagtatatctc R:gtacccgggaagctgctttgaataatt	$(CTAT)_{21}$	385			

[a] Applied Biosystems (Foster City, California) 5′ fluorescent phosphoramidite dyes: 6-Fam, Hex, and Ned.

TABLE 2. Number of females and males, proportion of polygynous males, number of all offspring, number of extra-pair offspring (EPO) detected out of total offspring genotyped, and the number of nests with at least one EPO out of the total number in which full clutches were genotyped for the population of Southwestern Willow Flycatchers in the Kern River Valley, California from 1995 to 2002.

Year	Females	Males	Proportion polygynous males	Number of offspring	No. EPO/No. genotyped	No. nests with EPO/total
1995	23	33	0.03	37	–	–
1996	28	29	0.17	53	–	–
1997	38	37	0.32	42	3/30	3/12
1998	25	30	0.13	52	11/50	4/19
1999	23	25	0.20	27	4/22	3/8
2000	12	25	0.08	14	2/13	2/5
2001	11	21	0.05	14	0/12	0/6
2002	13	18	0.11	19	0/14	0/6

$72°C$ for 15 sec), preceded by a 2-min incubation time at $94°C$, was repeated 35 times.

DETERMINATION OF PARENTAGE

Parentage was determined using either behavioral observations or genotypic analysis. By behavior, maternity was assigned to the individual that constructed the nest, incubated the eggs, and cared extensively for the young. The male that defended the territory in which the nest was located was considered to be the father. Therefore, based on behavioral data, all nestlings in a nest were assigned to the same male and female. Genetic parentage was determined by comparing nestling genotypes to the territorial male and female genotypes at eight loci. Territorial males were excluded as sires if their genotype did not match that of a nestling at more than one locus. In cases with single locus mismatches, the territorial male was excluded only if the genotype of another male provided a perfect match. If a resident male was unknown or excluded, paternity was only assigned if a perfect genotypic match was made between the offspring and an after-hatch-year male. Extra-pair males with perfect genotypic matches could be detected for 80% of extra-pair offspring. Total exclusionary powers of 0.980 (first parent) and 0.998 (second parent) were calculated using CERVUS 2.0 (Marshall et al. 1998).

STATISTICAL ANALYSES

To compare reproductive success between monogamous and polygynous males using behavioral data, the mean number of fledglings produced per season between 1995 and 2002

was calculated. Genetic comparisons of reproductive success were based on the subset of nestlings from which blood samples were taken and included extra-pair offspring lost through extra-pair fertilizations as well as EPO gained through extra-pair copulations. Comparisons are reported as mean ± SE. Two-tailed Mann-Whitney U-tests were used to compare reproductive success between treatments. Because one extra-pair fertilization can result in more than one EPO in a nest, we also compared polygynous and monogamous males in terms of the number of nests that contained at least one EPO using Fisher's exact test.

RESULTS

MATING STRATEGIES

Between 1995 and 2002, the proportion of behaviorally polygynous males ranged from 0.03 to 0.32 (mean = 0.14 ± 0.03; Table 2). The proportion of females that paired with polygynous males ranged from 0.09 to 0.71 (mean = 0.36 ± 0.07). From 1997 through 2002, between 69% and 96% of young produced in this population were genotyped and examined for extra-pair paternity. During this time, the total number of extra-pair offspring we detected each year varied from 0 to 11 (0%–21% of individuals genotyped), while the proportion of nests with at least one EPO ranged from 0%–40% (Table 2).

EFFECTS OF MATING STRATEGIES ON REPRODUCTIVE SUCCESS

Reproductive success for males that were behaviorally polygynous versus those that were behaviorally monogamous was calculated in-

dependent of genetic parentage based on 258 offspring from 143 nests monitored between 1995 and 2002. Based on these data, polygynous males fledged more offspring (mean = 3.81 ± 0.51) than monogamous males (mean = 1.74 ± 0.18; $U = 1819$, $n_1 = 78$, $n_2 = 32$, $P < 0.001$), but approximately the same number of offspring per pair (polygynous pairs: mean = 1.87 ± 0.19; monogamous pairs: mean = 1.74 ± 0.18; $U = 2689$, $n_1 = 78$, $n_2 = 65$, $P = 0.53$).

Genetic data were obtained from both parents and full clutches of 56 nests from 1997 to 2002 (Table 2). These data showed that even when losses to extra-pair offspring were included, polygynous males again fledged more genetic offspring (mean = 2.11 ± 0.35) than monogamous males (mean = 1.15 ± 0.18; $U = 1111$, $n_1 = 61$, $n_2 = 28$, $P = 0.02$), but approximately the same number of genetic offspring per pair (polygynous pairs: mean = 1.23 ± 0.19; monogamous pairs: mean = 1.15 ± 0.18; $U = 1531$, $n_1 = 61$, $n_2 = 48$, $P = 0.68$). Including those EPO that were known to have been sired by either monogamous or polygynous males in estimates of realized reproductive success did not alter the finding that polygynous males sired more young. When additions due to EPO were included, polygynous males sired more offspring (mean = 2.18 ± 0.37) than monogamous males (mean = 1.30 ± 0.22; $U = 1090$, $n_1 = 61$, $n_2 = 28$, $P = 0.04$), but approximately the same number of offspring per pair (polygynous pairs: mean = 1.23 ± 0.26; monogamous pairs: mean = 1.30 ± 0.16; $U = 1502$, $n_1 = 61$, $n_2 = 48$, $P = 0.82$).

Sires could be assigned to 16 of the 20 extra-pair offspring we detected; one polygynous male sired two EPO in one nest in a territory approximately 75 m away, two monogamous males sired eight EPO in two nests (~75 m and ~15 m away), one unpaired territorial male sired one EPO in a nest approximately 3.8 km from his territory, and four males that were never documented exhibiting territorial behavior (presumably nonterritorial floaters) sired five EPO in four nests. In all nests with EPO, only one male other than the resident male contributed offspring. There was no significant difference in the number of nests that contained EPO sired by monogamous (5 of 30) or polygynous males (7 of 27; Fisher's exact test, $P = 0.54$). Nonterritorial males were harder to detect and sample than territorial males; as

a result, they likely sired the remaining four EPO with unidentified paternity. Nests containing the EPO with unidentified sires were located in areas where all nearby territorial males were sampled. Thus, the sires of these EPO were either distant territorial males or nonterritorial (floater) males that were never captured and sampled.

DISCUSSION

We found no evidence that polygynous males had higher or lower numbers of extra-pair offspring in their nests than monogamous males. Previous studies that have found that polygynous males raised higher numbers of EPO (Bollinger and Gavin 1991, Dunn and Robertson 1993, Freeland et al. 1995, Soukup and Thompson 1997, Pilastro et al. 2002, Hamao and Saito 2005) have often argued that this may be due to difficulty in guarding multiple mates simultaneously (the "trade-off" hypothesis; Westneat et al. 1990, Birkhead and Møller 1992, Soukup and Thompson 1997). Our results suggest that since Willow Flycatcher males are relatively free from the demands of nest building, incubation, and nestling care, the ability to guard against EPO is no different for monogamous or polygynous males. Polygynous males may avoid EPO if their mates are reproductively receptive asynchronously, so that they are able to guard each mate sequentially (Hamao and Saito 2005). Unfortunately, we were unable to effectively test this hypothesis in our system, in part due to the large number of nests that were unsuccessful, with eggs or nestlings disappearing before we could collect DNA. Although we could potentially estimate overlap in female receptivity for these nests, our inability to determine whether they contained EPO left us with little discriminatory power.

The alternative hypothesis, that polygynous males should raise fewer EPO due to female preference for high-quality polygynous males (the "female choice" hypothesis; Westneat et al. 1990, Kempenaers 1994), was also not supported. Females behaviorally mated with polygynous males were just as likely to have EPO as those behaviorally mated with monogamous males. In all of the clutches that contained EPO in our study, only one extra-pair male fathered young in any given nest. This could have resulted if EPO in each nest were the result of

a single extra-pair fertilization, with variable numbers of young resulting from that single insemination, or if EPO were the result of multiple extra-pair fertilizations with the same male. In either case, there was no evidence that females were attempting to maximize genetic input to broods by seeking copulations from multiple extra-pair males.

Our analysis indicates that polygynous, monogamous, unmated, and nonterritorial males all participated in extra-pair interactions in this population. Regardless of mating strategy, most territorial birds sired EPO in nests relatively close to their own territory (<75 m away), as has been shown for several other species (Westneat 1993, Hasselquist at al. 1995, Thusius et al. 2001, Webster et al. 2001, Byers et al. 2004). The one exception was an unmated male whose territory was nearly 4 km from the nest containing his offspring. The fledgling sired by this male was in a nest initiated in the first week of July, after females had settled onto territories. Observational data confirmed that this male abandoned his territory, a typical behavior for unmated males during the beginning of July (MJW, unpubl. data). Males outnumbered females in all but one year of our study, and at least some males were polygynous in each year, so nonterritorial or unmated males were always present. Given this surplus of males, it is perhaps surprising that the percentage of EPO remained so low. Still, floater males were identified as sires of EPO in four of the six years of this study, indicating that these males do realize at least low levels of reproductive success.

Overall, this analysis indicates that previous studies using behavioral estimates of parentage were essentially correct; polygynous Southwestern Willow Flycatcher males achieve higher reproductive success than monogamous males and this is not offset by higher rates of extra-pair fertilization. This conclusion depends on numbers of EPO documented in the Kern River population being representative of other populations of Southwestern Willow Flycatchers. Prevalence of EPO has been shown to vary both in space and time in other bird species (Westneat and Mays 2005) and changes in density have been hypothesized to play an important role in some of these species (Birkhead 1978, Westneat et al. 1990, Møller and Birkhead 1991, Thusius et al. 2001). The small number of EPO we documented in each year of this study precluded us from testing the importance of these parameters. However, given the dynamic nature of nesting habitat for Willow Flycatchers, and often marked annual variation in population size, determining how prevalence of EPO responds to demographic changes in this species remains an important question.

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