

NESTLING SEX RATIO IN THE SOUTHWESTERN WILLOW FLYCATCHER

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Abstract. Using molecular-genetic techniques, we determined the gender of 202 Southwestern Willow Flycatcher (*Empidonax traillii extimus*) nestlings from 95 nests sampled over a five-year period. Overall nestling sex ratio did not vary significantly from 50:50 among years, by clutch order, or by mating strategy (monogamous vs. polygamous pairings). However, we did observe significant differences among the four sites sampled, with sex ratios biased either toward males or females at the different sites. Given the small population sizes and geographic isolation of many of the endangered subspecies' breeding populations, sex-ratio differences may have localized negative impacts.

Key words: *Empidonax traillii*, endangered species, nestling sex ratio, Willow Flycatcher.

Cociente de Sexos en Polluelos de *Empidonax traillii extimus*

Resumen. Utilizando técnicas moleculares determinamos el sexo de 202 polluelos de *Empidonax traillii extimus* pertenecientes a 95 nidos que fueron muestreados durante un período de cinco años. En general, el cociente de sexos no varió significativamente de 50:50 entre años, ya sea por orden de la nidada o por estrategia de apareamiento (monogamia vs. poligamia). Sin embargo, observamos diferencias significativas entre los cuatro sitios muestreados, en los cuales la razón de sexos estuvo sesgada hacia los machos o las hembras dependiendo del sitio. Dados los pequeños tamaños poblacionales y el aislamiento geográfico de muchas de las poblaciones reproductivas de esta subespecie en peligro, las diferencias en el cociente de sexos pueden tener un impacto negativo a nivel local.

Most avian species studied to date have shown a nestling sex ratio close to 50:50 at the time of hatching (Clutton-Brock 1985, Krackow 1995). Some studies, however, have detected nestling sex-ratio biases, and in some cases linked a skewed ratio to one or more ecological or behavioral traits (Sheldon 1998). Sex-ratio bias can occur at egg laying (primary sex ratio), at hatching (secondary sex ratio), or at fledging (tertiary sex ratio). A few studies have shown an apparent ability of females to adjust the primary sex ratio, although the mechanism for this is unclear (Krackow

1995, Komdeur et al. 1997). However, tertiary sex-ratio adjustment is the most commonly observed phenomenon and presumably results from differential parental care, such as favoring survival of the smaller, less costly sex to raise during times of limited resources (Cronmiller and Thompson 1987). Clutch or laying sequence can also consistently skew the sex ratio in some species (Ryder 1983, Weatherhead 1985). However, some species with ecological traits that might favor an adaptive sex-ratio bias (e.g., nest helpers, polygyny) show no bias toward a particular sex (Sheldon 1998).

Although recently receiving increased research attention, nestling sex ratios have been examined for only a small number of species, with a focus on those with sexually dimorphic nestlings. Gowaty (1993) noted the need for more studies, particularly of sexually monomorphic species where biases are hard to detect. The advent of molecular-genetic sexing techniques allows such monomorphic species to be more readily studied, which will provide more information on how common (or rare) sex-ratio biases are, and which environmental or ecological factors are most likely to result in variations in the sex ratio, if any. Furthermore, an understanding of nestling sex ratios is of particular interest for endangered species. A skewed nestling sex ratio may affect the adult population's sex ratio, especially in small and isolated populations; this in turn can affect breeding strategy, genetic variation (by reducing effective population size), and ultimately population persistence.

The Willow Flycatcher (*Empidonax traillii*) is a sexually monomorphic Neotropical migrant that breeds across most of the conterminous United States into southern Canada, and winters from southern Mexico to northern South America. It is highly territorial and typically monogamous, though polygyny does occur at some sites (Sedgwick 2000). The Southwestern Willow Flycatcher subspecies (*E. t. extimus*) is a federally endangered species that has declined in numbers over the last 100–150 years (USFWS 1995). It breeds in dense, mesic riparian habitats, and consequently now exists primarily in small (usually <10 breeding adults), often geographically isolated breeding sites across the southwestern United States (Marshall 2000). Willow Flycatchers will move to other breeding sites, although most individuals return to the same breeding site in subsequent years, and banding studies (Luff et al. 2000) indicate that movement between sites decreases with increasing geographic distance. Thus, the Southwestern Willow Flycatcher is a species where unequal

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nestling sex ratios could have important ramifications on populations (particularly isolated populations) and their long-term viability. As part of a larger genetic study (Busch et al. 2000, Paxton 2000), we collected DNA from flycatcher nestlings and determined their gender using molecular-genetic techniques. Concurrent demographic field studies allowed us to determine if sex ratios varied due to egg or nestling loss, yearly variation, mating strategy, clutch order, or breeding site differences.

METHODS

From 1996 to 2000, we collected blood from nestling Southwestern Willow Flycatchers at four sites in Arizona: Roosevelt Lake (Gila County; 33°46'N, 111°15'W), the San Pedro River (Pinal County; 32°53'N, 110°43'W), the Verde River (Yavapai County; 34°34'N, 111°51'W), and the White Mountains (Apache County; 33°50'N, 109°08'W). These four sites are 68 to 290 km apart. We located nests during the nest-building or egg-laying stages and monitored nests to determine clutch size, hatching success, and nesting success (Paradzick et al. 2001). We monitored color-banded adults to determine the order of successive nesting attempts and whether a particular nesting attempt was part of a monogamous or polygamous mating. Nestlings were banded with a single federal bird band between 7 to 10 days of age; DNA samples were obtained at the time of banding. To obtain blood for DNA analysis, we clipped the toenail just past the vascularized tissue to release a single drop of blood (Busch et al. 2000), which we washed into a vial with a blood buffer (1× SSC, 50 mM EDTA) and kept cool or on ice until it could be frozen at the end of the day.

GENETIC ANALYSIS

DNA was isolated from the blood following the procedure described by Mullenbach et al. (1989) with modifications described in Busch et al. (2000). We used Fridolfson and Ellegren's (1999) molecular sexing technique to determine the gender of nestlings. This technique utilizes the presence of the CHD-gene on the female sex-linked chromosome and a non-sex-linked chromosome, such that females have two different-sized bands when the PCR amplicon is run on an electrophoresis gel, while males have only one. Samples with ambiguous results were rerun. The technique was verified for accuracy by testing over 200 samples from adults of known sex (determined by presence of a brood patch or cloacal protuberance at time of capture); we observed less than a 1% discrepancy between the two methods, which in all cases were determined to be field-sexing errors. Polymerase chain reactions (PCR) consisted of 50 ng of DNA, 1× PCR buffer, 3 mM MgCl₂, 200 μM of dNTPs, 1 μM of each primer (2550F: 5'-GTTACTGATTCGTCTACGAGA-3' and 2718R: 5'-ATTGAAATGATCCAGTGCTTG-3'; Fridolfson and Ellegren 1999), and 1 U of *Taq* DNA polymerase, with 35 cycles of 30 sec at 94°C, 30 sec at 55°C, and 2 min at 72°C. PCR products were run on a 1.5% agarose gel, stained with ethidium bromide, and scored manually to determine the gender.

STATISTICAL ANALYSIS

For our initial examination of the data, we used SPSS 10.1 (SPSS Inc. 1999) software to compute chi-square statistics to test for deviations from the expected nestling sex ratio of 50:50 (unity) in comparisons of primary vs. secondary or tertiary sex ratios, years, sites, mating strategy, and clutch order. However, because the broods, not the nestlings, are considered the independent units when evaluating nestling sex ratios (Gowaty 1991), we also analyzed the sex ratios using a general linear model (GLIM 4.09; Numerical Algorithms Group 1994) with binomial errors and a logit link (Crawley 1993). This method takes into account that the variances are binomially distributed and that the variance and sample size vary from brood to brood. The size of the discrepancy between the model and the data is denoted by the deviance, which is distributed asymptotically as chi-square. The significance of the various factors we tested for was determined by the change in the deviance of the null model (ΔD) as the factors were added. Significant variation in the sex ratio among broods (departure from the binomial distribution) was determined using the deviance in the null model, and significance was determined using a chi-square table. We accepted statistical significance if $P \leq 0.05$. All sex-ratio percentages are expressed as percent female.

RESULTS

We determined the gender of 202 nestlings from 95 clutches (Table 1). Clutch size ranged from 1 to 4, with a mean clutch size of 2.7 (mean number of nestlings sampled per clutch was 2.1). Initial examination of the data indicated numerical departures from equal nestling sex ratios in the dataset, although a chi-square analysis indicated only a single site (Roosevelt Lake) was significantly different from 50:50 ($\chi^2_1 = 5.2, P < 0.05$). For all subsequent analyses, we tested the binomial distribution of sexes within the broods. To examine whether the observed sex ratios were a result of egg or nestling mortality, we tested for differences between those clutches with no mortality and those where the number of sampled nestlings was less than the initial clutch size. Forty-five clutches had the same number of nestlings sampled as the initial clutch size (providing a primary sex ratio), 32 clutches had a single egg or nestling reduction, and nine had two eggs or nestlings missing (providing secondary or tertiary sex ratio). Because we were not always able to determine whether a reduction from the initial clutch size was at the egg or nestling stage, we lumped both types of losses together. There was no significant difference between primary sex ratios and subsequent sex ratios (Table 2); therefore, we pooled both groups for subsequent analysis. Sex ratio did not deviate from 50:50 when considering the factors of year, mating strategy (monogamous vs. polygamous), or clutch order (first vs. subsequent clutches). Although the sex ratio of all sites combined was not significantly different from 50:50, individual sites were skewed toward either males (two sites) or females (two sites), with the site factor being significantly different from unity ($\Delta D = 16.8, df = 3, P < 0.001$; Table 2). Evaluating sites individually indicated that the proportion of females varied signif-

TABLE 1. Number of and sex ratios of nestling Southwestern Willow Flycatchers; ratios are presented by egg or nestling mortality,^a clutch order, mating strategy, and breeding site. Missing information for some factors resulted in differing sample sizes per grouping.

Factor	Group	No. female	No. male	% female	No. of clutches
Nestling mortality	No mortality	59	55	52	45
	Mortality	45	26	63	41
Clutch order	First	67	59	53	59
	Second	43	31	58	34
Mating strategy	Polygamy	42	38	53	36
	Monogamy	47	33	59	37
Breeding site	Roosevelt	78	53	60	62
	San Pedro	18	27	40	22
	White Mts.	15	6	71	8
	Verde River	0	5	0	3
Total of all nestlings		111	91	55	95

^a Nestling mortality is defined as a reduction in number of nestlings compared with initial clutch size due to the loss of eggs or nestlings.

icantly among broods for the San Pedro River (null model = 33.9, $df = 21$, $P < 0.05$) and for the White Mountains (null model = 14.7, $df = 7$, $P < 0.05$), but not for Roosevelt Lake (null model = 75.6, $df = 61$, $P = 0.1$). We did not test the Verde River site due to small sample size (Table 1).

DISCUSSION

Although we detected significant sex-ratio biases, the Willow Flycatcher displays none of the traits typically associated with facultative sex-ratio manipulation. Sex-ratio biases have generally been associated with ecological traits such as nest helpers, sexually different rates of nestling growth, sexual dimorphism (especially size of nestlings), obligate polygyny, and differential dispersal among sexes (Clutton-Brock 1985). Flycatchers are primarily monogamous and show no evidence of helping at the nest (Sedgwick 2000). Male and female nestlings and adults are similar in plumage and size (though females are slightly smaller; Sedgwick 2000), and male and female dispersal and between-year movements are similar in distance (Luff et al. 2000, Sedgwick 2000). Consistent with this was the overall nestling sex ratio, which did not differ with egg or nestling mortality, clutch order, or mating strategy.

TABLE 2. Significance of primary vs. secondary or tertiary sex ratio (nestling mortality), year, clutch order, mating strategy, and breeding site as factors explaining variation in the sex ratio of Southwestern Willow Flycatcher broods. Significance was based on the degree to which the factors explained departures of the observed sex ratios from a binomial distribution.

Factor	df	ΔD	P
Nestling mortality	1	3.0	0.08
Year	4	2.8	0.59
Clutch order	1	0.5	0.48
Mating strategy	1	0.9	0.34
Breeding site	3	16.8	<0.001

The four sites combined resulted in a roughly equal sex ratio (55% female), although individual sites were skewed, and the site factor explained a highly significant amount of the variation. Roosevelt Lake and the White Mountains were skewed toward females while the San Pedro River and the Verde River were skewed toward males. When the other factors were applied to each site, we observed no departure from 50:50. Differential sampling of nestlings among sites over the five-year period may have obscured a time effect (year sampled); however, most sites were sampled in most years, and the effect of year poorly explained the observed variance (Table 2). Thus, there appeared to be significant variation among sites that none of the factors we evaluated could explain. Furthermore, although the differences are intriguing, we know of no ecological factors that would promote this varying bias among sites. Site-by-site variations in the sex ratio may be due to chance, small sample size, changes in the population size, or a consequence of small breeding populations. Inbreeding has been suggested as a possible mechanism for sex-ratio biases (Frankham 1995), but Busch et al. (2000) reported that Willow Flycatchers in the Southwest retain substantial genetic variability.

Banding studies (Luff et al. 2000) show that flycatchers disperse and move between the Arizona study sites, but not in sufficient numbers to change the overall sex ratio of breeding adults at these sites. Fledglings are also more likely to return to their natal site than to disperse (Kenwood and Paxton 2001). Thus, a locally skewed nestling sex ratio could lead to imbalances in the local adult population's sex ratio, as has been shown for the endangered Roseate Tern (Szczyg et al. 2001). The White Mountains, with a small breeding population and nestling ratio of 71% females, has been declining in size since 1996; extreme polygyny observed there (up to four females to a male) may be due to a surplus of females. Other flycatcher studies have noted apparent unequal sex ratios in small populations along the Colorado River in the Grand Canyon (where 44% of territorial males were unmated;

Sogge et al. 1997), and on the Rio Grande in New Mexico (Johnson et al. 1999) where a lack of females was one explanation for a high proportion of unpaired territorial males. Whether these apparent adult sex-ratio imbalances are due to an unequal nestling sex ratio for these areas is unknown.

Because the Southwestern Willow Flycatcher is endangered, deviations from a 50:50 nestling sex ratio may be of concern for other reasons as well. Modeling of endangered populations (e.g., estimates of population growth and population viability analysis) often incorporates nestling sex ratios as a parameter assumed to be 50:50. Stoleson et al. (2000) modeled Southwestern Willow Flycatcher population growth assuming that the sex ratio of nestlings was equal; a bias toward males would depress population growth estimates while a bias toward females would increase estimates (S. Stoleson, pers. comm.). Furthermore, incorporating demographic stochasticity of sex ratios is an important consideration for PVA models because the absence of this will underestimate extinction probabilities (Brook et al. 2000). Our data indicate that it may be acceptable to assume equal nestling sex ratios at a large scale (e.g., the state of Arizona), but that unequal sex ratios could lead to inaccurate model estimates at the regional or site level. The draft Southwestern Willow Flycatcher recovery plan (USFWS 2001) recommends focusing recovery efforts on "management units" that are based on portions of major drainages; at this scale, sex-ratio equality cannot be assumed and modelers or managers should take into account how nestling sex-ratio variation could affect models and local population stability.

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