EVIDENCE FOR A CASE OF MULTIPLE PATERNITY IN THE RED BAT (*LASIURUS BOREALIS*) AS INDICATED BY DNA FINGERPRINTING

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Crichton and Krutzsch (2000) recently summarized the body of literature detailing the reproductive biology of bats. However, many aspects of the reproductive biology for most chiropteran species are still poorly known (Racey, 1988), because their nocturnal and reclusive behavior often precludes direct observation of courtship and mating.

Females of some of the more readily observed social building/cave dwelling bat species have been observed to mate with many males (McCracken and Wilkinson, 2000, and references cited therein). However, issues of multiple paternity will be few for an order of mammals that typically produces but a single offspring following such multiple matings (Nowak, 1999; Racey and Entwistle, 2000). The few previous DNA fingerprinting studies of bats were intended to provide data supplemental to observations of nursing strategies or mating patterns of communal roosters (*Pipistrellus pipistrellus*, Bishop et al., 1992; *Myotis lucifugus*, Watt and Fenton, 1995; Petri et al., 1997),

although we are aware of one application of this technique (*Nyctalus noctula*, Mayer, 1995) in which dizygotic twins may be fathered by different males.

The red bat (*Lasiurus borealis*) is considered a monotypic species (Baker et al., 1988; Genoways and Baker, 1988), and is seasonally one of the more common of bat species across much of its range. Yet, it is a surprisingly little understood species that is perhaps best known for its atypically (for a bat) large litter sizes of 3-4 young (Barbour and Davis, 1969, Stangl et al., 1996). Several attributes of its life history typical for the genus contribute to its elusive nature: the bat is a solitary tree rooster that is often difficult to locate; migratory patterns and population structure are poorly understood; and little is known of its mating behavior beyond a few anecdotal observations.

The red bat may be a year-around resident in other parts of the species' range, but it is transient in north Texas. Local populations during the spring

months of April and May are comprised exclusively of pregnant females, having arrived from parts unknown. Most births occur in early June, and both mothers and grown young have vacated the region by August. This study documents levels of inter- and intra-litter genetic variation for five north Texas family groups of *L. borealis* that afford valuable insight into reproductive behavior of the species.

METHODS AND MATERIALS

Procurement of Specimens.—Seventeen specimens of *Lasiurus borealis* were collected and described by Stangl et al. (1996). All individuals originated from within the city limits of Wichita Falls, Wichita County, Texas. Museum vouchers (MWSU catalog numbers) reside in the Collection of Recent Mammals, Midwestern State University (MWSU), and tissues (TK reference numbers) were deposited in the frozen tissue banks of The Museum, Texas Tech University.

Respective reference numbers and familial relationships of animals are as follows: one adult female (MWSU 14884, TK 29661) with three offspring (MWSU 14885-14887; TK 29658-29660); another adult female (MWSU 18035, TK 29921) with three offspring (MWSU 18206-18208; TK 29922-29924); and litters of four (MWSU 16719-16722; TK 29838-29841), three (MWSU 16728-16730; TK 29844-29846), and two (MWSU 15851, 15852; TK 29748, 29749) siblings.

Extraction, Electrophoresis, and Hybridization of DNA.—DNA was extracted by a modification of the technique described by Gemmell and Akiyama (1996). The heart and both kidneys of each specimen were collectively weighed and placed in a single 1.5 ml microcentrifuge tube containing 300 µl of digestion buffer (100 mM NaCl, 50 mM Tris-HCl/pH 8.0, 1% SDS, 50 mM EDTA/pH 8.0). Proteinase K was added to a final concentration of 100 µg/ml and each tube was incubated at 50 °C for 2 h., then at 37 °C overnight (16-18 h).

RNase A was added to a final concentration of 1 mg/ml and incubated for 30 min at 37 °C. One buffer volume (300 µl) of 5M LiCl was added and each sample was mixed by inversion for 1 min. Samples were extracted with two buffer volumes chloroform by gentle mixing on a rotary shaker for 30 min followed by centrifugation for 15 min at 14,000 x g. DNA was ethanol-precipitated from the aqueous phase and pelleted by centrifugation for 5 min at 14,000 x g. The supernatant was decanted and DNA pellets were washed with 800 µl of 70% ethanol and centrifuged again for 3 min. The supernatant was decanted and DNA pellets were air-dried for 10 min and resuspended in 200 µl TE/pH 7.76. Samples were refrigerated overnight at 4 °C, and were periodically shaken by hand to dissolve DNA.

Absorbance values at 260 nm and 280 nm were determined with an Ultrospec III (Pharmacia) spectrophotometer. Twelve micrograms DNA from each specimen was digested at 37 °C for 6 h with 5 u *Hinf I*/µg DNA in a final volume of 75 µl. DNA was precipitated by addition of 7.5 µl of 3M NaOAc and 150 µl of 95 % ethanol prior to cooling on ice for 20 min. Samples were centrifuged at 14,000 x g for 20 min, and the supernatant was decanted. DNA pellets were air-dried for 10 min and resuspended in 30 µl of TE/pH 8.0. Twenty microliter (8 µg) samples were loaded onto a 30 x 25 cm 0.8% agarose gel with HiLo® molecular weight markers (Minnesota Molecular, Minneapolis) and electrophoresed at 100 V for 30 min, and then at 50 V for 16 h.

DNA was transferred to a nylon membrane (Duralon, Stratagene) by descending capillary transfer overnight, and the membrane was air-dried for 30 min and baked in a vacuum oven (20 in Hg) at 80 °C for 2 h. The membrane was prehybridized in a shaking waterbath for 1 h at 42 °C with 20 ml of UltraHyb hybridization solution (Ambion) and hybridized with 200 ng of a 1 kbp poly (dA-dC) x poly (dG-dT) probe fluoresced with the Prime-It Fluor kit (Stratagene). Hybridization was accomplished at 42 °C in a shaking waterbath for 21 h.

Detection and Analyses of Bands.—The membrane was removed from the hybridization bag and washed for 15 min at room temperature in 2x SSC/

1% SDS, 15 min at 60 °C in 2x SSC/1% SDS, and 15 min at 60 °C in 0.5x SSC/1% SDS. Fluoresced hybrids were detected with the Illuminator Chemiluminescent Detection System (Stratagene, 1997), with blocking at 45 °C. The processed membrane was sealed in plastic, exposed to X-ray films (CEA, Strängnäs, Sweden) for 30 min, 2 h, and 3 h, and developed manually.

The site of a band in any one or more samples of a family group (representing either sibling or mother) was treated as a distinct phenotype. Each sample of that family group was scored on the presence or absence of a band at that specific locus, regardless of band intensity or the possibility of "allelic" variation (differential migration rates). Distinct bands in each sample were numbered from largest (~10 kb) to smallest (~3 kb). Below 3 kb, background exposure made scoring unreliable. Band comparisons were made within individual family groups, and no inference was made regarding the homology of numbered bands between litters due to variation in migration rates.

The proportion of shared bands as a measure of genetic similarity (S) between individuals within family groups were expressed using the equation:

$$S = 2N / (N1 + N2),$$

where N is the number of bands shared by both individuals, and N1 and N2 are the total number of bands recorded for each (Lynch, 1990). The resulting similarity matrix was analyzed phenetically using the Unweighted Pair Group Method (UPGMA) algorithm. All computations were accomplished with the NCSS 97 statistical package (Hintze, 1997).

RESULTS

The number of detectable loci in the 3-10 kb size class varied between family groups. Extremes ranged from 26 (family groups 1 and 5) to 34 for litter 3 (mean of 28; Figure 1, Appendix 1). The proportion of shared bands that were scored for any given litter varied considerably, and no two individuals exhibited a fingerprinting pattern the same as either mother (when available) or siblings. One specimen (S1 of litter 5) possessed each of the 26 bands scored for that litter of two, while another animal (S4 of litter 3) expressed little more than half (19 of 34) of the total number of bands recorded for that litter of four siblings.

The mean genetic similarity (S) between siblings of each litter was 0.80 (Table 1, Appendix 2), although a phenetic portrayal of similarity indices demonstrated considerable variation both among and between litters (Figure 1). DNA fingerprints of the two litter 5 siblings were nearly identical (S=0.94), and pairwise comparisons between siblings of litters 1, 2, and 4 were comparable (respective means [and range] of pairwise comparisons were: 0.85 [0.83-0.87], 0.86 [0.82-0.95], and 0.83 [0.77-0.87]). Among the four siblings of litter 3, the mean similarity index was 0.63 (range of 0.50-0.80). Mean similarity values of mothers to offspring were comparable to sibling-to-sibling indices (0.77 for litter 1; 0.84 for litter 2).

DISCUSSION

Evidence for Multiple Paternity

Obtaining definitive answers to the determination of paternity in multiple-birth litters with limited sample sizes and the lack of potential paternal (and in some cases, even maternal) genotypes is not likely. One can deduce paternity with varying degrees of reliability by matching male-presumptive bands with potential fathers, but our data (only two litters with mothers; N = 3 for each litter; no potential fathers) were insufficient to permit this approach. Nevertheless, several observations provide strong circumstantial evidence for the likelihood that litter 3 was sired by more than one male (and possibly as many as three).
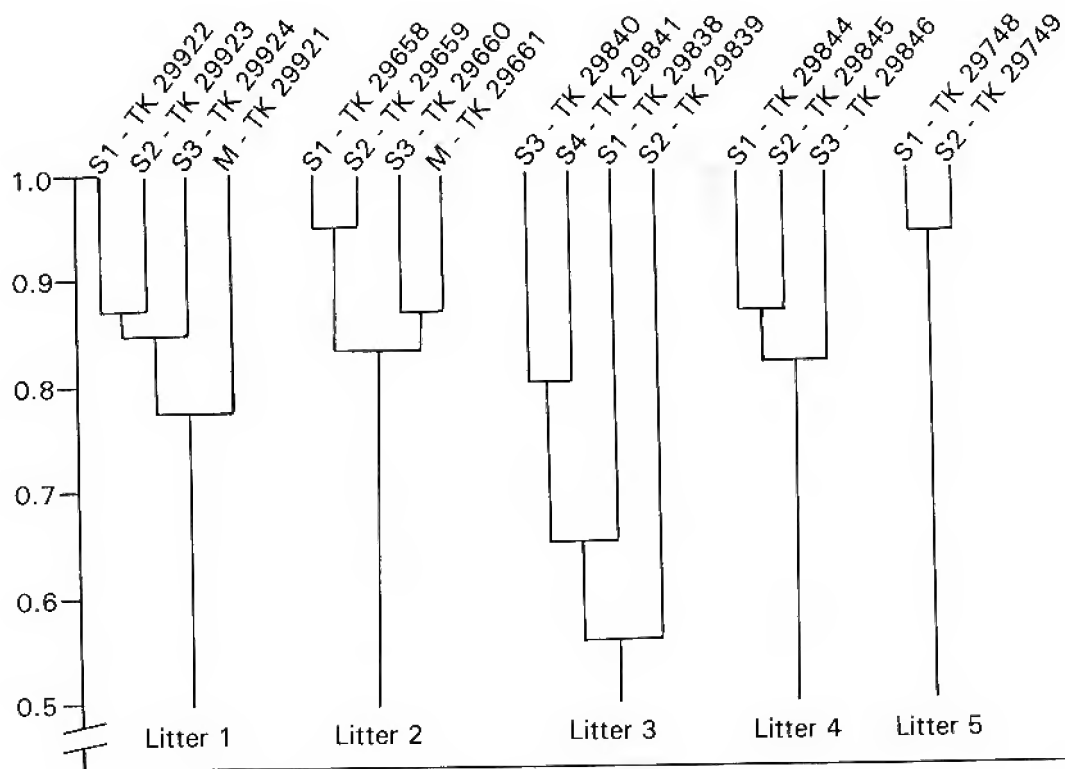
Figure 1. UPGMA phenograms of 17 specimens representing five family groups of *Lasiurus borealis*. Tissue voucher (TK) numbers are provided for numbered siblings (S) of each litter, and, where obtained, mothers (M).

Table 1. Interlitter variation for siblings of five litters of Lasiurus borealis, based on DNA fingerprinting data. Descriptive statistics are: sample size (N), range, mean, standard deviation (SD), similarity index (S), confidence interval (C. I.), and coefficient of variation (C. V.).

Siblings (N)	Similarity index			
	Range	Mean ± SD	95% C. I.	C. V.
Litter 1 (3)	0.83-0.87	0.85 + 0.02	0.80-0.90	2.35
Litter 2 (3)	0.82-0.95	0.86 + 0.06	0.71-1.00	6.98
Litter 3 (4)	0.50-0.80	0.63 + 0.10	0.53-0.73	15.87
Litter 4 (3)	0.77-0.87	0.83 + 0.04	0.73-0.93	4.81
Litter 5 (2)	0.94	0.94	—	—

Comparative levels of genetic variation.—Each of the litters (exclusive of litter 3) demonstrated comparable levels of genetic similarity, exceeding 80% levels of similarity between confamilial members. DNA fingerprints for siblings of litter 3 demonstrated far higher levels of variation (mean S of 0.63) than those of other family groups. Statistical evidence for the genetically distinctive nature of litter three is compelling: the 95% confidence intervals for similarity indices among siblings in litter 3 exhibit almost no overlap with those of other litters, and the coefficient of variation for litter 3 is twice to more than five times that of any other litter. Such levels of genetic variation are sufficient to postulate that more than one male was involved in the fostering of litter 3.

DNA fingerprints for the red bat (*Lasiurus borealis*) demonstrate a higher level of similarity for first-order relationships within family groups (sibling to sibling, or mother to sibling) in relation to comparable data for a taxonomically diverse contingent of other small mammals. Similarity indices for first-order relationships (sibling-sibling, mother-offspring) among *L. borealis* generally ranged in the vicinity of 0.80 (Table 1), in contrast to *Sorex araneus* (mean of 0.65; Tegelström et al., 1991), *Myotis lucifugus* (range of 0.22-0.42; Watt and Fenton, 1995), *Sciurus carolinensis* (range of 0.77-0.88; David-Gray et al., 1998), *Cynomys gunnisoni* (range of 0.41-0.67; Travis et al., 1997), *Dipodomys spectabilis* (mean of 0.56; Keane et al., 1991), *Peromyscus californicus* (range of 0.43-0.59; Ribble, 1991), *Peromyscus leucopus* (range of 0.33-0.61; Schug et al., 1992), *Sigmodon hispidus* (range of 0.46-0.48; Descalzi et al., 1998), *Microtus ochrogaster* (range of 0.64-0.79; Hoagland et al., 1991), *Microtus pinetorum* (mean of 0.59; Marfori et al., 1997), and *Microtus montanus* (mean of 0.57; Cummings and Hallett, 1991).

The two taxa demonstrating values most comparable to those for *L. borealis* were based on specimens from populations having experienced population bottlenecks via human manipulation: *S. carolinensis*, from European gray squirrel populations which trace their ancestry on that continent to a series of introductions, of unreported sample sizes and of unspecified geographic origins, from the United States between 1876 and 1929; and *M. ochrogaster*, from a laboratory colony of prairie voles maintained at the University of Kansas.

We do not discount sampling error due to the relatively small sample size as a causative factor for the observed high levels of genetic similarity represented in the DNA fingerprints of *L. borealis*, but the red bat is presently understood to be a monotypic species—exhibiting no racial variation of morphological or genic characters warranting subspecific designations (Baker et al., 1988; Genoways and Baker, 1988).

Morphological congruence.—The most convincing evidence for multiple paternity in litter three is the congruent phenotypic variance of the litter 3 siblings, as contrasted to uniformity in growth rates typical for the species. Of 34 nursing young red bats representing 12 litters, Stangl et al. (1996) noted that variation in size and development between siblings of the same sex is negligible. A notable exception was a litter that exhibited a remarkable variation in size and stages of development. Stangl et al. (1996) remarked that: "Given the rather uniform rates of development between siblings of the same sex and of different ages in the other litters, this does not appear to represent a normal variance that might be attributed to individual rates of development." This litter corresponds to the genetically variable litter 3. A reexamination of their data (Table 2) demonstrates that the coefficient of variation of individual weights (in g) for this litter of four males (17.24) is far greater than that recorded for six other litters (mean of 4.63, range of 2.35-8.88). It must be noted that five of the other six litters with lesser similarity values are of mixed sex, and that sexual dimorphism in size, even from birth, is evident in red bats.

Stangl et al. (1996) postulated that differential rates of development or varied timing in embryonic implantation might account for this phenomenon. Supported by the highly individualized DNA fingerprints of litter 3 siblings, it is not unreasonable to postulate multiple paternity as an explanation for the observed variance in size and development. If this scenario is true, then the mother of litter 3 must have experienced early spring copulation with two or more males, with implantation events likely occurring at intervals concordant with time passed between separate matings

Table 2. Morphological data and sex composition (m = male, f = female) for seven litters of immature Lasiurus borealis *reported by Stangl et al. (1996), arranged in order of relative age. Descriptive statistics are: mean, standard deviation (SD), and coefficient of variation (C.V.). Asterisk (*) for litter d indicates the genetically variable litter #3, as determined by DNA fingerprinting.*

Litter (N: sex composition)	Weight range (g)	mean±SD	C.V.
Litter a (4: 2m, 2f)	1.5-1.6	1.53±0.04	2.84
Litter b (3: 3f)	2.6-2.8	2.70±0.08	3.02
Litter c (4: 1m, 3f)	3.4-3.6	3.53±0.08	2.35
*Litter d (4: 4m)	3.4-5.6	4.58±0.80	17.24
Litter e (3: 2m, 1f)	6.4-7.4	6.70±0.50	7.41
Litter f (3: 2m, 1f)	8.4-9.1	8.73±0.29	3.28
Litter g (3: 2m, 1f)	8.4-10.2	9.07±0.81	8.88

with different males. Synchronized birth of siblings with varied gestation times (e.g. time elapsed from conception to birth) would account for the apparently varied rates of development. However, the question of whether or not multiple matings with a single male preceeded the conception for single-sired litters remains unanswered.

ACKNOWLEDGMENTS

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APPENDIX 1

Scoring of DNA fingerprint bands for 17 specimens of Lasiurus borealis *representing five family groups comprised of siblings (S) and mothers (M). Numbering system of bands does not imply homology for across-litter comparisons. Numbered bands are presented sequentially from the 3- to 10-kb range.*

Numbered bands	Litter 1				Litter 2				Litter 3				Litter 4			Litter 5	
	M	S1	S2	S3	S1	S2	S3	M	S1	S2	S3	S4	S1	S2	S3	S1	S2
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
2	1	1	1	1	1	0	1	1	1	1	0	0	1	1	0	1	1
3	0	0	1	1	0	0	1	1	1	1	1	1	1	0	1	1	1
4	1	1	1	1	1	1	1	1	1	0	0	0	0	1	0	1	1
5	1	0	0	1	1	1	1	1	0	1	0	0	1	1	1	1	1
6	1	0	0	1	1	1	1	0	0	1	1	1	1	1	1	1	0
7	0	1	1	1	1	1	1	1	1	0	1	1	0	1	0	1	1
8	1	1	0	1	1	1	1	1	1	0	0	0	1	0	1	1	1
9	1	0	0	1	1	1	1	1	0	1	1	1	1	1	1	1	1
10	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1	0
11	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1
12	1	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1
13	0	0	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1
14	0	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1
15	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1
16	1	0	1	1	0	0	0	1	1	1	1	0	1	1	1	1	1
17	0	1	1	0	1	1	1	0	1	0	1	0	1	1	1	1	1
18	1	1	1	1	1	1	1	1	0	1	0	1	1	1	1	1	1
19	1	1	1	1	0	0	1	1	1	0	1	0	0	1	1	1	1
20	1	0	1	0	1	1	1	1	0	1	0	1	1	1	0	1	0
21	1	1	1	1	0	0	1	1	1	0	1	1	1	0	1	1	1
22	1	1	1	1	1	1	1	1	0	1	0	0	1	1	0	1	1
23	0	1	1	1	0	1	1	1	1	0	1	0	0	0	1	1	1
24	1	1	1	1	1	1	0	1	1	1	1	1	1	1	0	1	1
25	1	1	1	1	0	0	1	0	0	0	1	1	1	1	1	1	1
26	1	1	1	1	1	1	0	1	1	1	0	0	1	1	1	1	1
27	-	-	-	-	1	1	1	1	1	1	1	0	1	1	1	-	-
28	-	-	-	-	-	-	-	-	1	1	1	1	-	-	-	-	-
29	-	-	-	-	-	-	-	-	0	1	1	1	-	-	-	-	-
30	-	-	-	-	-	-	-	-	1	0	1	1	-	-	-	-	-
31	-	-	-	-	-	-	-	-	0	1	0	0	-	-	-	-	-
32	-	-	-	-	-	-	-	-	1	0	0	0	-	-	-	-	-
33	-	-	-	-	-	-	-	-	0	1	0	0	-	-	-	-	-
34	-	-	-	-	-	-	-	-	1	1	1	1	-	-	-	-	-

APPENDIX 2

Sorensen's similarity indices and resulting correlation matrices for five family groups of Lasiurus borealis, *based on DNA-fingerprinting data in Appendix 1.*

	Litter 1				Litter 2				Litter 3				Litter 4			Litter 5	
	M	S1	S2	S3	S1	S2	S3	M	S1	S2	S3	S4	S1	S2	S3	S1	S2
Litter 1																	
M	-	0.74	0.73	0.84	-	-	-	-	-	-	-	-	-	-	-	-	-
S1		-	0.87	0.83	-	-	-	-	-	-	-	-	-	-	-	-	-
S2			-	0.86	-	-	-	-	-	-	-	-	-	-	-	-	-
S3				-	-	-	-	-	-	-	-	-	-	-	-	-	-
Litter 2																	
S1					-	0.95	0.82	0.82	-	-	-	-	-	-	-	-	-
S2						-	0.82	0.82	-	-	-	-	-	-	-	-	-
S3							-	0.88	-	-	-	-	-	-	-	-	-
M								-	-	-	-	-	-	-	-	-	-
Litter 3																	
S1									-	0.50	0.73	0.57	-	-	-	-	-
S2										-	0.57	0.60	-	-	-	-	-
S3											-	0.80	-	-	-	-	-
S4												-	-	-	-	-	-
Litter 4																	
S1													-	0.87	0.86	-	-
S2														-	0.77	-	-
S3															-	-	-
Litter 5																	
S1																-	0.94
S2																	-

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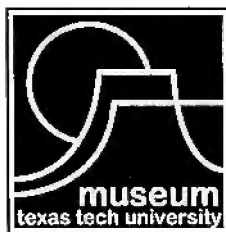*email: frederick.stangl@mwsu.edu*

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