Anim. Behav., 1993, **46**, 847–860

Evening bat isolation calls provide evidence for heritable signatures

J. ANDREW SCHERRER* & GERALD S. WILKINSON†
Department of Zoology, University of Maryland, College Park, MD 20742, U.S.A.

(Received 11 August 1992; initial acceptance 12 August 1992; final acceptance 18 September 1992; MS. number: A6319R)

Abstract. Vocalizations of infant evening bats, *Nycticeius humeralis*, have unique characteristics that contain sufficient information for a mother to distinguish her offspring from other offspring in a colony. Using seven acoustic variables, all 306 isolation calls recorded from 39 pups in one attic nursery colony at 2–5 days of age were classified to the correct pup without error. Studies of captive bats reveal that the amount of among-bat variation in call variables remains constant from birth through to at least the next 14 days. Though several variables change as pups grow, an individually distinctive pattern of frequency modulation can be recognized at all ages. Isolation calls also contain information about family identity, as expected when call characteristics are heritable: calls of wild pup twins were assigned to the correct family 68% of the time and three variables had significant heritabilities between 0·61 and 0·85. Factor analysis indicates that infant isolation calls exhibit independent variation in at least four dimensions. Consequently, over 1800 pups could produce calls without overlap in any dimension of acoustic space.

In several bird (e.g. penguins, Jouventin 1982) and mammal (e.g. Mexican free-tailed bats, *Tadarida brasiliensis*, McCracken 1984) species, dozens to millions of young are left to intermingle in creches while their parents forage. For returning parents of these species to locate and provision their offspring without error, a distinctive signal or signature (Beecher 1982) must be produced by the young and both detected and recognized by the parent. Thus, for a signal to provide signature information, signal variation among individuals must exceed signal variation within individuals. In other words, signature efficacy is directly proportional to repeatability, which measures the amount of variation that occurs among rather than within individuals (Falconer 1981; Boake 1989).

The variation expected among offspring signature signals should increase whenever misdirected parental care lowers the future reproductive success of offspring. Such variation has four possible causal sources: (1) natural selection on heritable signals, (2) trial-and-error learning by young to avoid overlap with the signals of other young, (3) copying by young of unique parental signals or (4) parental labelling of young with unique cues. Because both trial-and-error learning and imitation require an initial learning period, genetically transmitted signals or parental labels are expected if discrimination must occur immediately after birth or hatching.

When signature signals occur to solicit parental feeding, kin recognition is possible. As Grafen (1990) has recently noted, true recognition of kin, rather than recognition of group or species, requires not only that recognition signals be variable but that they also impart information about the relatedness between signaller and receiver. If relatedness between parent and young takes multiple values within a group of offspring, heritable signatures can provide information about relatedness. To discriminate between offspring using heritable cues, parents must be able to compare their own signature signal to that of the offspring. For vocal signals this requires matching an infant's call to a maternal call or template.

Studies of vocal recognition in birds have shown that signature signals often develop at a time during ontogeny when intermingling of young becomes a potential problem (Hepper 1986). Colonial bats differ from most birds in that bat young intermingle immediately. Within the first day after birth, many adult female bats leave their pups in nursery creches while they go out to forage. This feature places more immediate demands on the recognition system of bats than of birds and suggests either that bat pups and their mothers learn signature signals very rapidly, or that differences between signature signals are largely governed by genetic factors.

*Biometrics Unit, Cornell University, Ithaca, NY 14853, U.S.A.
†To whom correspondence should be sent.

0003–3472/93/110847 + 14 $08.00/0

Bat pups utter contact-promoting calls known as isolation calls when separated from their mothers. These calls do not occur prior to parturition, but are uttered minutes after birth, often several days before the occurrence of echolocation-like calls (Gould 1971, 1975). If a newborn pup does not vocalize, its mother will not begin normal maternal care (Gould 1971). Most isolation calls consist of multiple harmonics, exhibit sinusoidal frequency modulation (FM) and are repeated in a series of 1–30 units but differ from linear FM sweeps used for echolocation in that they are usually longer (50–60 ms as compared with 5–10 ms), lower in frequency (10–30 kHz rather than 20–200 kHz), and more variable in frequency modulation (Fenton 1985). The sharp onset, repetitiveness, and tonal nature of these calls make them easy to locate (Marler 1959). Many workers (e.g. Brown 1976; Brown et al. 1983; Thomson et al. 1985; Gelfand & McCracken 1986; Jones et al. 1991) have suggested that bat isolation calls act as signatures and they closely resemble signature whistles given by the dolphin, *Tursiops truncatus* (Sayigh et al. 1990).

Although mother–infant reunions have been staged with several colonial bats and have demonstrated that mothers can use isolation calls (see review in Fenton 1985; Balcombe 1990) and smell (Gustin & McCracken 1987) to locate their offspring, how newborn bats acquire these cues has received little study. Because *Phyllostomus discolor* pups appear to converge on the FM patterns of distinctive maternal calls, or directive calls, several weeks after birth, Esser & Schmidt (1989) have suggested that pups learn to imitate the vocalizations of their mothers. However, such evidence is not incompatible with some or even most of the variation in isolation calls being heritable. If maternal directive calls are ontogenetically related to infant isolation calls as Brown (1976) has suggested, then maternal–offspring resemblance will result from genetic transmission. In this study we use variation in isolation calls produced by infant evening bats, *Nycticeius humeralis*, to determine whether they contain signature information and to identify features that remain characteristic of individuals during the first 2 weeks of ontogeny. Because evening bats produce two or sometimes three pups per litter, we also use the similarity in call variables between siblings to estimate genetic variation and covariation for call variables and show that individual differences in isolation calls are consistent with genetic transmission.

METHODS

Field Recordings

To obtain recordings we captured infant bats at four attic nursery colonies during June 1988 and 1989. The Grim colony was 6 km south of Pulaski, Iowa, the Zion colony 4 km east of Cainsville, the Hutton colony 6 km west of Cainsville, and the Busby colony 1 km north of Galt were in Missouri. We captured pups 2–5 days of age by hand in the roost at the Zion colony while adult females were foraging, and at the other colonies during the day while the pups were nursing. We usually kept pups out of the roost less than 1 h and recorded approximately 100 calls per pup. We assigned pups to sibship, at the Zion colony from nursing observations (Wilkinson 1992). We assumed pups that nursed from the same mother during the first 2 weeks after birth were siblings because we confirmed the maternity of most of the Zion pups through sequence comparisons of a 500 bp region of mitochondrial DNA (Wilkinson & Chapman 1991; Wilkinson 1992) and because communal nursing was not observed among pups less than 12 days of age (Watkins & Shump 1981; Wilkinson 1992). To provide a consistent recording environment we placed pups in a small sound-proof chamber made from a styrofoam cooler lined with eggcarton foam. Pups were kept a constant distance of 20 cm from the microphone by placing them in a small wire cage ($3 \times 4{\cdot}5 \times 6$ cm) inside the cooler. We recorded vocalizations on a Precision Instruments model 207 tape-recorder at 30 ips with a modified Granath ultrasonic microphone and amplifier (Simmons et al. 1979) and monitored the number of calls and microphone gain during recordings with a Tektronix model 214 portable storage oscilloscope.

Captive Recordings

During the summers of 1989 and 1990 we kept bats in captivity for two ontogenetic studies. In May 1989, we caught nine pregnant *N. humeralis* females from the Grim colony and 10 from the Hutton colony and maintained them in separate cages in a single room. Sixteen females gave birth to two young while three produced three young each. The pups from one female died shortly after birth and she was released. In May 1990, we captured 30 female evening bats in an attic in Edenton, North Carolina, and divided them into three separate

groups of 10 adults each. One Edenton female failed to give birth and one lost its litter just after birth. All Edenton litters contained two young except one that had only a single pup. In both years we captured pregnant females in their roosts 1–2 weeks prepartum, kept them for 6 weeks in cages measured 20 × 30 × 40 cm and lined with nylon screen to provide roosting surfaces, and then released them with their pups at the site of capture. Twice per day before pups were born and three times per day after birth, we fed adult females on mealworms that had been raised on vitamin-supplemented, high-protein cereal (Wilson 1988). All pups that survived the first day after birth appeared to grow and develop normally in captivity. We recorded isolation calls by removing pups to another room while mothers fed using the same techniques and equipment as described above for field recordings.

In 1989 we recorded 20 pups at 1, 2, 4, 7, 10 and 14 days of age. This recording schedule was designed to encompass the period of rapid growth when calls are expected to change most dramatically and include periods later in development when pups are beginning to expand their call repertoires and are more independent of their mothers (Gould 1971, 1975; Brown 1976). In 1990 pups were recorded four times in the first 2 days after birth in order to permit closer study of the changes that occur when bats are only a few days old. The pups used for this study consisted of five pairs that were born within 24 h of each other. We recorded these 10 pups at 6, 14, 26 and 40 h after parturition.

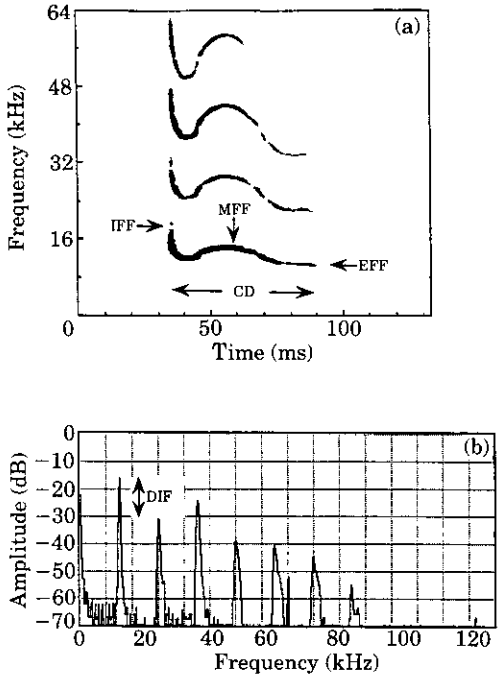

Figure 1. Frequency versus time (a) and amplitude versus frequency (b) plots of a typical pup isolation call, indicating the seven call variables measured. The variables are initial frequency of the fundamental (IFF), middle frequency of the fundamental (MFF), end frequency of the fundamental (EFF), difference in amplitude between the fundamental and the first harmonic measure at the IFF (IDIF), middle difference in amplitude (MDIF), end difference in amplitude (EDIF), and call duration (CD).

Sound Analysis

During recording sessions both wild and captive pups uttered FM sweeps as well as isolation calls. Two or three transitional calls were often uttered between groups of FM calls and isolation calls. We avoided these transitional calls in favour of unambiguous isolation calls. In all but four cases, calls measured for each pup were taken from different calling bouts within a sample to minimize temporal dependence among calls within a bout.

We transformed recorded calls played at 7·5 ips (i.e. slowed to one-fourth the original speed) into spectrograms using a Kay Elemetrics model 5500 digital sonagraph which sampled at 40·96 kHz and displayed a 16-kHz frequency range. Transform size was 512 points, yielding a time resolution of 1·5 ms and bandwidth of 117 Hz in real time.

To summarize spectral variation in isolation calls we measured frequency modulation, relative intensity and duration using seven variables from each call (Fig. 1). The pattern of frequency modulation in the call was determined by measuring frequency at the beginning, middle and end of the fundamental, noted hereafter as IFF, MFF and EFF, respectively. Although absolute intensity of calls was unreliable for comparisons among individuals, any consistent pattern of intensity differences between harmonics could reflect individual differences in emphasized harmonics caused by vocal tract resonance. Therefore, we also measured intensity differences in dB between the first two harmonics at the locations where frequency measurements were taken. These relative intensity variables are labelled IDIF, MDIF and EDIF, respectively. We identified onset or termination of a

call when the signal moved 20 dB above or below background and use CD to denote call duration.

Statistical Analysis

Variation in wild pups

For these analyses we selected seven to 12 calls from each of 78 pups (656 total calls) that were 2–5 days of age to control partially for age-related changes in call characteristics. We estimated ages of wild bats from a least squares regression of forearm length on age for 21 female and 19 male pups reared in captivity in 1989 (female age = 1·05 (forearm) − 13·06, $r^2 = 0{\cdot}87$; male age = 0·95 (forearm) − 11·04, $r^2 = 0{\cdot}92$). Each bat was measured six times between 1 and 14 days of age. Forearm length changes linearly with age for the first 20 days after birth (Jones 1967). We entered age as a covariate into all statistical models to remove further age effects.

We conducted two multivariate analyses of variance (MANOVAs) to determine whether males differ from females. One method used 277 calls from the 17 families where both pups were the same sex to determine whether families of males differed from families of females, on average. The second method was a split-plot analysis, with colony, family and pup on the whole plot, and sex on the sub-plot. In this analysis we used the 379 calls from 22 families with mixed-sex litters to test for differences within families due to sex. Because sex was not significant in either analysis, we ignored sex in all subsequent analyses.

Three linear discriminant function analyses (Johnson & Wichern 1988) were used to group observations by colony, family and pup, and to quantify the amount of overlap among calls from each of these nested groups. To classify calls to the correct pup within a colony, we used the seven variables measured on 38 individuals from the Busby colony to generate the discriminant function. To classify calls to the correct family we used five variables, which we discovered by factor analysis (see below) to be important in among-family variation, in a second discriminant function model that used the mean values for each of 19 families at the Busby colony. To classify calls to colony we created a third discriminant function using means of the seven variables from all 78 pups from all four colonies. These analyses were performed using a cross-validation procedure (SAS Institute 1989) in which each observation was classified using a discriminant function model computed from the other observations, excluding the observation being classified. This procedure reduces any bias introduced by using a model to classify observations that were used to create that model. The prior probability of classifying N_i observations correctly by chance is equal to $N_i/\Sigma N_i$, where N_i is the number of observations obtained for each group i.

To partition the variation observed in the call variables to each of the three levels of nesting we conducted two MANOVAs. The first model had families, pups and the error term corresponding to calls within pups as sources of variation. Since each of these factors were random effects, we used SAS type III sums of squares to generate both univariate and multivariate variance components. We then computed intra-class correlation coefficients (Sokal & Rohlf 1981) at the family and pup levels to estimate heritability, h^2, and repeatability, respectively (Falconer 1981; Lessels & Boag 1987; Boake 1989). The intra-class correlation among families provides an upper estimate of half the broad-sense h^2 (Falconer 1981). Our h^2-estimates could, therefore, be inflated by shared maternal effects or genetic interactions including dominance and epistatic variation.

To estimate phenotypic and genetic correlations we computed a second MANOVA in which calls for each pup were averaged, leaving families within colonies and pups within families as the two sources of variation. The genetic correlations were calculated from $(MS_{fam} - MS_{pup})/n$, and the phenotypic correlations were derived from MS_{pup}, where MS_{fam} and MS_{pup} are matrices containing the expected mean squares for call variables at the among family and among pup levels, respectively, and n is the number of pups per family (Becker 1975; Falconer 1981). Using 95% confidence intervals (Falconer 1981), we tested whether genetic correlations were significantly different from 0, 1 or −1 to determine whether two characters were independent or shared a common genetic basis.

In order to identify those call variables that contributed to the separation of the calls at each level of the nested model, the multivariate variance components were each analysed using a principal factor analysis with a varimax rotation. Two variables, MFF and MDIF, were excluded from the factor analysis at the among-family level. Attempts to include these variables in the analysis resulted in a negative definite covariance matrix due to variance estimates of less than zero.

Ontogenetic changes

We examined ontogenetic changes in isolation calls using analysis of covariance (ANCOVA) with pup as the main effect and age as a covariate. The linearity of the age effect was tested by fitting orthogonal linear, quadratic and cubic terms. Of the samples taken over 2 weeks, recordings from days 10 and 14 did not contain sufficient isolation calls for nearly half the pups, restricting the analysis to the data from days 1, 2, 4 and 7. Four of the pups produced isolation calls through to the first 4 days only and were omitted from the analysis; thus, this sample consisted of eight sibling pairs. Each pup was represented in both the 2-week and 2-day samples by four calls from each of four recording sessions, yielding totals of 256 and 160 calls, respectively. Although related pairs of pups were used in both samples, the nesting structure of pup within family was not used in either analysis because tests of family effects were not significant.

We used Levene's test for homogeneity of variances (Milliken & Johnson 1984) to determine whether variation in each call variable changed with age. Levene's method consists of obtaining the absolute values of the residuals from the ANOVA model of interest, and subsequently using these values as data in the same ANOVA model. If the resulting F-statistic is significant, variances are not homogeneous. For examination of trends in within-pup variation, the absolute residuals from all calls in the ANCOVA (above) were used as data for another ANCOVA. In order to look at among-pup variation, we used the absolute residuals from the means of all the observations for each pup in a similar ANCOVA. In both analyses, absolute residuals were $\log_{10}$ transformed to satisfy normality and homoscedasticity assumptions. For each case, we conducted multivariate analogues of Levene's test to determine whether the overall variation in the call changed over time.

All statistical analyses used SAS version 6·06 (SAS Institute 1989).

RESULTS

Isolation Calls

Most *N. humeralis* isolation calls decreased in frequency from beginning to end through one cycle of a sinusoidal wave (Fig. 1). The mean ($\pm$SE) IFF for the 78 wild pups 2–5 days of age was $18{\cdot}8 \pm 0{\cdot}4$ kHz, while average MFF and EFF were $15{\cdot}6 \pm 0{\cdot}4$ and $14{\cdot}3 \pm 0{\cdot}5$ kHz, respectively. Standard errors were calculated from the mean parameters of one pup per family to avoid underestimating the population variance due to resemblance among relatives. The mean ($\pm$SE) intensity difference between the first and second harmonics was $13{\cdot}8 \pm 0{\cdot}9$ dB at the beginning, $9{\cdot}1 \pm 1{\cdot}1$ dB in the middle and $10{\cdot}1 \pm 1{\cdot}5$ dB at the end of the call. Call duration averaged ($\pm$SE) 53 ± 2 ms, but some isolation calls were as short as 36 ms or as long as 73 ms. The FM sweeps that pups produced during this period were shorter (6–18 ms) than isolation calls, but still considerably longer than those of adult bats (3–5 ms). The frequency range of FM sweeps was comparable to that of isolation calls produced at the same age.

Variation among Pups

After removing linear effects due to age, ANOVAs and MANOVAs revealed significant variation among pups. Variance component estimates (Table I) for each of the call variables indicated that the proportion of total call variation that was attributable to differences among pups averaged over 50% and equalled as much as 81% for MFF. These among-pup differences reflect striking similarity of calls within individuals as demonstrated by significant repeatabilities for all seven variables (Table II). The discriminant function model successfully classed 100% of the calls to the correct pup within the Busby colony (Table III). These results indicate that there is no multivariate overlap among the isolation calls of different individuals within a colony.

On average, pups exhibited a progressive increase in frequency parameters, as well as a significant decrease in call duration (Fig. 2) over the first 7 days. Changes with age are best described as linear, since quadratic and cubic terms were not significant. No significant change in intensity differences between the first and second harmonics occurred with maturity, in either the 7-day (Fig. 2) or the 2-day sample. For pups recorded in the first 2 days, the trend toward higher frequency was indicated only by an increase in IFF between 6 and 14 h ($P = 0{\cdot}003$). Although duration decreased between 6 and 14 h (63 ± 8 to 51 ± 4 ms), this difference was not significant. Nevertheless, the MANOVA indicated an overall effect of age ($P = 0{\cdot}003$) for pups recorded during the first 2 days.

The changes in call variables of different individuals were not parallel over time, based on a sig-

Table I. *F*-tests from nested ANOVAs on the seven call variables measured as in Fig. 1

	Colony		Family		Pup		Call
Variable	*F*	VCE†	*F*	VCE†	*F*	VCE†	VCE†
IFF	1·76	0·06	2.52**	0·37	48·1***	0·49	0·09
EFF	3·32*	0·15	1·83*	0·23	85·5***	0·57	0·06
MFF	0·50	0·00	1·29	0·07	56·9***	0·81	0·12
IDIF	2·55	0·06	1·59	0·12	8·3***	0·38	0·43
EDIF	0·73	0·00	1·99*	0·23	13·9***	0·47	0·30
MDIF	1·43	0·01	1·32	0·06	6·3***	0·36	0·56
CD	1·10	0·00	2·06*	0·30	38·1***	0·57	0·13
Average‡		0·04**		0·20***		0·52***	0·24
N§		4		39		78	656

*$P<0·05$; **$P<0·01$; ***$P<0·01$.
†Variance component estimates (VCE) show proportion of variation explained by differences among colonies, families within colonies, pups within families, and calls within pups.
‡Significance determined from Wilk's lambda.
§*N* indicates sample size at each level.

Table II. Heritability and repeatability estimates, and their standard errors for seven isolation call variables (see Fig. 1) from 39 pairs of wild pup siblings

	Heritability	SE	Repeatability	SE
IFF	0·85*	0·26	0·91*	0·01
EFF	0·76*	0·27	0·94*	0·01
MFF	0·15	0·32	0·88*	0·02
IDIF	0·37	0·31	0·57*	0·05
EDIF	0·45	0·30	0·70*	0·04
MDIF	0·15	0·31	0·44*	0·05
CD	0·61*	0·29	0·87*	0·02

*95% confidence interval does not include zero.

nificant interaction between pup and age effects (Wilk's lambda, $P=0·0001$), for both 2- and 7-day samples. However, even though frequencies and duration change during development, spectrograms taken over a 14-day period (Fig. 3) illustrate that the pattern of frequency modulation in an individual's call remains consistent during at least the first 2 weeks of ontogeny.

If imitation of siblings occurs, then variation among isolation calls within pups should decrease as pups age. Similarly, trial-and-error learning that causes unrelated pups to become dissimilar will increase variation among pups as they age. In contrast, little change in variation over time is expected if most call differences are heritable. The multivariate test for homogeneity of variances indicated no significant overall change in the amount of variation among calls within individuals for the 7-day sample (Levene's test, Wilk's lambda, $P=0·55$) or for the 2-day sample (Levene's test, Wilk's lambda, $P=0·71$). Furthermore, the overall amount of variation also remained constant among bats for both the 2-day and the 7-day samples (Wilk's lambda, $P=0·58$, $P=0·07$, respectively). Results of univariate tests for each variable agreed with those of MANOVA, except in one case for the 2-day among-bat sample, where the amount of among-bat variation in CD decreased (Wilk's lambda, $P=0·0011$).

Variation among Families

Calls exhibited variation among families beyond that found among pups within families (Fig. 4, Table I). On average, differences among families explained 20% of the overall call variation; however, only four of the variables, IFF, EFF, EDIF and CD, were significant at the family level. Three of these call variables, IFF, EFF and CD, showed significant h^2 estimates (Table II). As expected, those variables that had the highest h^2 also displayed the highest repeatabilities. The frequency and relative amplitude variables measured at the middle of the call, MFF and MDIF, exhibited the lowest repeatabilities and were not significant at the family level. Calls were assigned to the correct family 68% of the time by a discriminant function

Table III. Cross-validation results of linear discriminant function analyses

Assignment task	Total no. observations	Total no. classes	Prior probability†	% Correctly assigned
Calls to bat	306	39	0·026	100
Calls to family	306	19	0·053	68
Calls to colony	656	4	—	56

†Prior probabilities for each colony: Busby = 0·487, Grim = 0·128, Hutton = 0·205, Zion = 0·180.

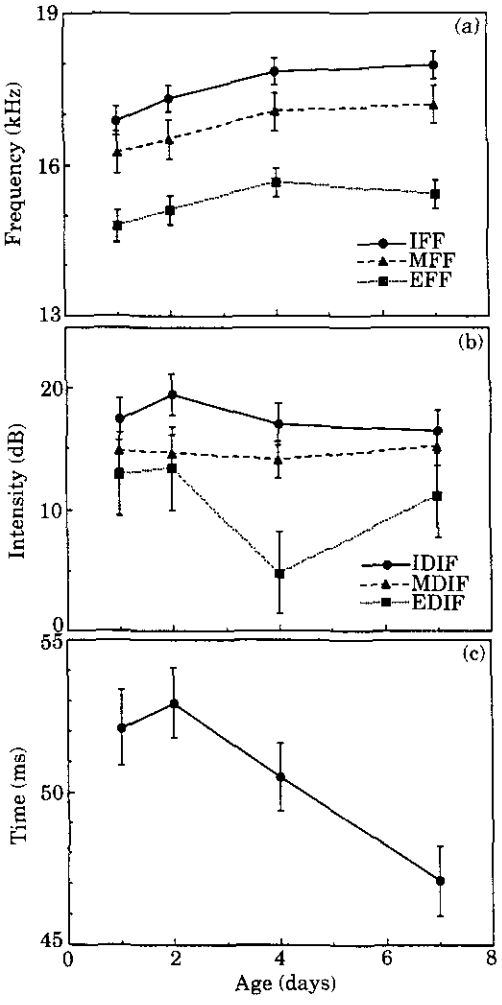

Figure 2. Plot of mean (±SE) frequencies (a), amplitude differences (b), and call durations (c) for 16 pups recorded in captivity on 1, 2, 4 and 7 days after birth. Refer to Fig. 1 for description of call variables.

model created from the mean values of each pup (Table III).

Genetic correlation estimates (Table IV) reflect the correlations among characters between pups within families. Six of the 21 genetic correlations were significantly different from one, thereby rejecting the hypothesis that these characters represent the same genetic factor. Of these six, four included IFF. Furthermore, most of the genetic correlations were similar in sign and magnitude to the corresponding phenotypic correlations. The exception of this tendency was IDIF, which correlated differently with EFF, MFF, EDIF and MDIF at the family level than at the individual level.

The hierarchical nested factor analysis revealed that some of the variables important for distinguishing among families were different from those which distinguished among pups (Table V). Furthermore, call features showed different loading patterns at the family and pup levels than at the call level. The end frequency and relative amplitude, EFF and EDIF, loaded predominantly into factor one for both the among-pup and among-family levels. For factor two, only the initial frequency loaded heavily at the among-family level while MFF and CD loaded heavily at the among-pup level. Four factors accounted for less than 90% of the explained variation at either the among-pup or within-pup levels. The factor analysis at the within-pup level revealed that those call variables measuring frequency (IFF, EFF and MFF) loaded into factor one, while those variables relating to call intensity (IDIF, EDIF and MDIF) loaded most heavily into factors three, four, and two, respectively. Duration information was important in the first three factors.

Variation among Colonies

Even after accounting for variation at the family and pup levels, a small (4%) but significant amount

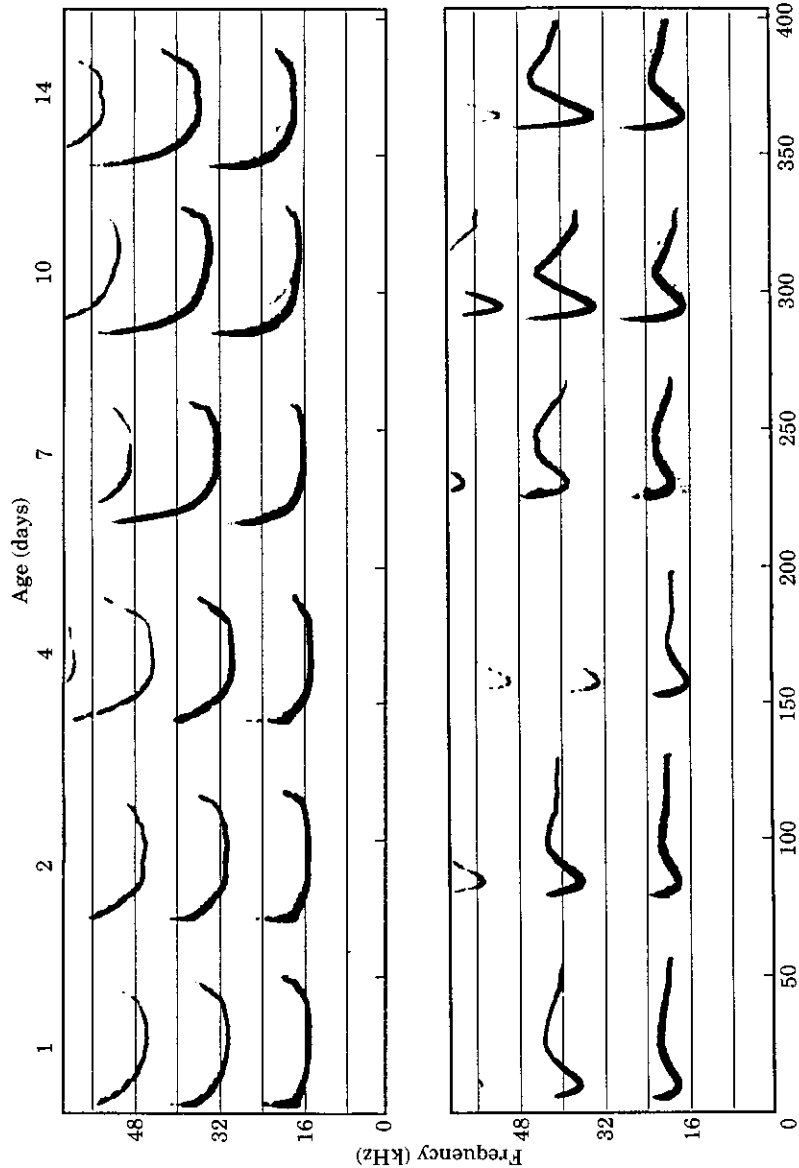

Figure 3. Series of typical spectrograms for each of two unrelated pups from the same colony on 1, 2, 4, 7, 10 and 14 days after birth showing changes in isolation calls through 2 weeks of ontogeny.

Table IV. Genetic (upper diagonals) and phenotypic (lower diagonals) correlations among call variables measured in wild pups

	IFF	EFF	MFF	IDIF	EDIF	MDIF	CD
IFF		0·262†	0·376	0·327†	0·139†	−0·002	0·011†
EFF	0·277		0·615	0·245†	0·895*	0·390	0·102
MFF	0·397	0·561		0·465	0·520	0·522	−0·098
IDIF	0·288	0·097	−0·170		0·256	0·714	0·140
EDIF	0·182	0·730	0·409	0·166		0·486	0·165†
MDIF	0·027	0·138	0·381	0·350	0·255		0·107
CD	−0·211	−0·014	−0·205	0·098	0·065	−0·004	

Call variables defined as in Fig. 1.
*95% confidence interval does not include zero.
†95% confidence interval does not include 1 or −1.

Table V. Varimax-rotated loadings of call variables (as in Fig. 1) onto the first four factors for the among-family, among-pup, and within-pup levels of variation

Variable	Factor 1	Factor 2	Factor 3	Factor 4
Among-families*				
IFF	0·097	**0·961**	0·108	0·233
EFF	**0·867**	0·156	0·177	0·382
IDIF	0·339	0·288	0·007	**0·894**
EDIF	**0·927**	0·048	0·282	0·154
CD	0·278	0·108	**0·954**	0·014
Cumulative†	0·57	0·78	0·93	0·99
Among-pups				
IFF	0·200	−0·416	−0·073	**0·793**
EFF	**0·951**	−0·205	0·058	−0·036
MFF	0·465	**−0·615**	**0·514**	−0·025
IDIF	−0·082	0·389	0·284	**0·798**
EDIF	**0·948**	0·025	0·109	0·139
MDIF	0·090	−0·009	**0·977**	0·119
CD	−0·034	**0·825**	0·008	−0·017
Cumulative†	0·38	0·59	0·74	0·88
Within-pups				
IFF	**0·816**	0·070	0·116	−0·072
EFF	**0·779**	−0·036	−0·038	0·219
MFF	0·733	0·393	0·025	0·044
IDIF	0·103	0·078	**0·888**	−0·012
EDIF	0·101	0·099	0·001	**0·965**
MDIF	−0·017	**0·773**	0·350	0·181
CD	−0·347	**−0·635**	0·374	0·052
Cumulative†	0·33	0·49	0·63	0·75

The two highest scores are shown in bold for each factor at each level, if greater in magnitude than 0·5.
*MFF and MDIF were removed from analysis due to zero variance (see text).
†Cumulative proportion of variation explained.

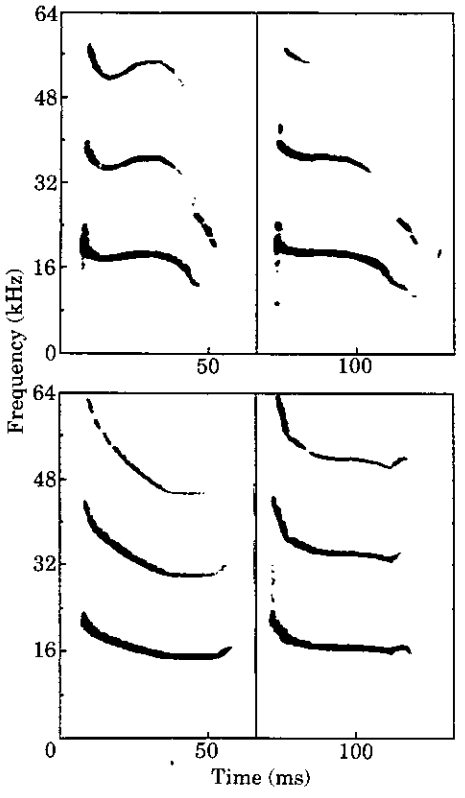

Figure 4. Four spectrograms illustrating a typical isolation call from each sibling in two different families from the same colony. Same-age siblings are paired from left to right.

of variation was explained by colony (Table I). ANOVA revealed that EFF was the only variable for which among-colony differences exceeded zero. The discriminant function model correctly identified the colony from which a call originated 56% of the time which was significantly greater than expected by chance (Table III).

DISCUSSION

Evidence for Signatures

For isolation calls to be useful as signatures they must (1) be variable among and repeatable within individuals, (2) contain diagnostic properties that persist through development, and (3) be readily perceived and recognized by lactating females. In this study we have provided data that confirm the first two requirements and below we provide support for the third requirement using additional observations and studies on other vespertilionid bats. *Nycticeius humeralis* isolation calls closely resemble those given by other vespertilionids, for example, *Eptesicus fuscus*, *Myotis lucifugus* (Gould 1971), *Antrozous pallidus* (Brown 1976), and *Pipistrellus pipistrellus* (Jones et al. 1991).

Although several previous studies have demonstrated individual differences in colonial bat isolation calls (e.g. Brown 1976; Barclay et al. 1979; Schmidt et al. 1982; Brown et al. 1983; Gelfand & McCracken 1986; Jones et al. 1991), age effects could have contributed to or masked differences among pups in all but one study (Jones et al. 1991). When we removed age as a covariate from our discriminant function model, calls were correctly assigned to pup in only 60%, rather than 100%, of cases. By conducting a series of nested analyses we also discovered that information about family and colony, in addition to pup, are contained in isolation calls. The MANOVA and hierarchical factor analysis results indicate that different call characteristics could be used to better distinguish among families than among pups and could, therefore, convey information about relatedness. Relatedness does not, however, predict the occurrence of communal nursing, which can account for up to 20% of nursing bouts late in lactation (Wilkinson 1992).

The lack of change in variation both among and within pups during the first 2 weeks after birth indicates that the repeatability estimates calculated for 2- to 5-day-old pups are representative throughout this period and possibly longer. The observed multivariate interaction between pup identity and age in both the 2- and 7-day samples also indicates that the spectral properties of isolation calls follow a unique trajectory for each pup. Even though calls, on average, increase in frequency and decrease in duration, any single parameter of an individual's call does not follow a predictable pattern. Thus, mothers cannot rely on a general rule for predicting the changes in a particular parameter. We suspect, therefore, that females are not responding to the absolute values of any of these features in isolation, but rather they must pay attention to the relative frequency modulation pattern of an individual's call, which remains constant as the bat ages (Fig. 3). Similar frequency modulated patterns have been reported to persist during development for other bats including *A. pallidus* (Brown 1976), *Desmodus rotundus* (Schmidt et al. 1982), *Noctilio albiventris* (Brown et al. 1983), *Phyllostomus discolor* (Rother

& Schmidt 1985), and *E. fuscus* (Moss 1988). Although Jones et al. (1991) suggest that infant *Pipistrellus pipistrellus* have signatures that change over time, their conclusion rests on comparing discriminant function loadings at two different ages. If call characteristics do not change linearly with age, then such loadings will change. Thus, their result does not preclude the possibility that signature shape is retained while spectral markers of that shape change allometrically with age.

The results of perceptual discrimination and neurophysiological experiments suggest that vespertilionid bats can detect much smaller differences in time, frequency, and intensity of returning echoes than needed to discriminate among pup isolation calls. For example, *E. fuscus* are able to detect echo arrival time differences of 29–41 μs (Simmons & Grinnell 1988). Because the theoretical maximal frequency discrimination is equal to the reciprocal of the duration of the signal (Simmons et al. 1975), the 20–30 ms constant frequency tail of an isolation call could permit discrimination of 33–50 Hz. Recent neurophysiological studies on *E. fuscus* indicate that the fine frequency discrimination associated with the neuronal filters of some constant frequency (CF) bats may also occur in vespertilionid frequency-modulated (FM) bats. Casseday & Covey (1992) have discovered inferior colliculus neurons that are tuned to 1-kHz bands between 20 and 30 kHz. The smallest detectible echo intensity difference for *E. fuscus* has been estimated to be between 1·5 and 3·0 dB (Simmons & Vernon 1971). Furthermore, Simmons (1973) found that *E. fuscus* can distinguish between holes of different depths in objects suggesting that vespertilionid bats can make judgements based on acoustic absorption spectra; i.e. the relative intensities of harmonics within an echo. For comparison, among-pup differences are on the order of 2–3 kHz in frequency, 5–10 dB in inter-harmonic intensity differences, and 5–10 ms in call duration. Perceptual sensitivity studies using communication calls as stimuli are needed to determine whether the auditory capabilities of bats attempting to discriminate between calls from different individuals differ from those used to process echoes.

Mother–infant reunions have also been simulated in two playback experiments to eliminate possible confounding effects of odour differences. Although the field playback study using *M. lucifugus* (Thomson et al. 1985) had low female responsiveness and the *T. brasiliensis* used in the captive study (Balcombe 1990) were tested in an unnatural environment, both studies demonstrate that females selectively respond to their own pup's isolation calls.

Several additional observations indicate that *N. humeralis* females must be able to use auditory cues to locate and distinguish young. Communal nursing never occurred during the first 10 days after birth (Wilkinson 1992) when females typically approach and retrieve calling pups. As pups age, they call less often and actively approach females to nurse. While females almost certainly use olfactory cues (Watkins & Shump 1981) and position to aid in identifying young, neither of these cues would allow females to locate and retrieve young that we placed several metres from where they were left before a female departed to forage. However, on all 22 occasions that we observed females in the Zion attic retrieve young after we had handled and moved them, the female was subsequently verified as the pup's mother. Furthermore, on two occasions we attempted to cross-foster pups less than 6 h after birth. At this age the pup's eyes and pinnae are closed. Even though both a foreign pup and a female's own pup were first rubbed with 70% ethanol, the female forcibly removed the foreign pup when it was placed in contact with her but allowed her own pup to nurse. Because both pups were calling vigorously, we surmise that females use vocal, rather than olfactory, cues to recognize their own young. Because they had relatively little time to learn those cues, these observations also suggest that females either rapidly imprint on their pup's calls or have a predetermined estimate of the call for comparison. Such a template is feasible only if call variation is highly heritable.

Given our results we estimate that sufficient variation exists to permit completely non-overlapping isolation calls for all pups in even the largest colony containing over 1000 *N. humeralis* (Watkins 1970). By using the range in each variable and the coefficients of the first four factors from the among-pup factor analysis (Table V), we can estimate the total acoustic four-dimensional space occupied by pup calls. Then, by using the average range for each variable within pups we can calculate the number of pup units spanned by the observed range in each of the four dimensions. The product of those units provides an estimate of the number of pups that could be packed into the existing four-dimensional acoustic space without allowing any call overlap. The number of pup units is 6·3, 9·5, 11·8 and 2·6

for factors one through four, respectively. Thus, if females use information from the first four factors, 1844 pups could produce completely unique isolation calls.

Evidence for Heritable Variation in Isolation Calls

Three lines of evidence suggest that few, if any, signature characteristics in isolation calls are learned by infant *N. humeralis*. (1) Because *N. humeralis* pups often produce isolation calls while still attached to the umbilical cord (personal observation), little time is available to learn a distinctive call after birth. In contrast to *A. pallidus* (Brown 1976), *P. discolor* (Esser & Schmidt 1989) and *T. brasiliensis* (Balcombe & McCracken 1992), we have never heard female *N. humeralis* utter directive calls, and antiphonal calling between mother and young does not occur as it does in *Rhinolophus ferrumiquinum* (Matsumara 1979, 1981). Thus, *N. humeralis* cannot learn isolation calls from their mothers. Furthermore, those newborn vespertilionid bats studied to date (Woolff 1973; Gould 1975; Brown et al. 1978) show no behavioural or neurophysiological responses to auditory stimuli. Behavioural responses to sound at birth have only been described for phyllostomatid bats (Brown et al. 1983; Reubsamen 1988; Esser & Schmidt 1990). (2) If pups learned their signatures by becoming different from other pups, then among-pup variation should have increased and within-pup variation decreased as pups had a chance to practice calls between 6 and 48 h after birth. While some modification of call parameters occurs during this period, the frequency modulation pattern representing the signature was unaltered and six of the call variables showed no change in variability. (3) Beginning the first night after birth *N. humeralis* females leave their young in tight clusters containing two to 30 similar-aged pups while they forage. Thus, newborn pups could imitate isolation calls from other members of a colony in addition to those of their siblings. If imitative learning of other pups occurred, then all pups in a colony might come to resemble each other. However, sufficient differences exist between pups 2–5 days of age to allow perfect assignment of every call to the correct pup and there is no decrease in variation between calls with age. Imitative learning among pups could, in fact, be maladaptive because it would tend to eliminate signature information.

The MANOVA results indicate that isolation calls do contain a small amount of information about colony identity. Such information is unlikely to be the result of genetic differences between colonies. Although mothers return to the same roost every year (Watkins 1970; Watkins & Shump 1981), the average level of relatedness within colonies is essentially zero because males apparently mate randomly at hibernation sites with females from several nursery colonies and mortality is high (Wilkinson 1992). Thus, call differences between colonies may indicate weak imitative learning or some other local environmental effect that influences call production. Note, however, that colony level differences account for only 4% of the overall variation in pup isolation calls.

Because the response to selection on a group of traits is influenced by both genetic correlations and heritabilities of the traits (Falconer 1981), those genetic correlations not significantly different from zero indicate pairs of call traits that can change independently, while those pairs that are not independent will exhibit correlated responses to selection. Isolation calls that differed in several independent acoustic dimensions could not only accommodate more unique signatures but could also respond to selection more rapidly than call characteristics that were all genetically correlated. Six genetic correlations involving four different variables were significantly different from 1 or -1. Thus, at least four genetic variables could evolve independently to create different signatures among individuals.

The conclusion that isolation call variation reflects genetic differences must be tempered with the realization that our estimates of genetic parameters come from comparing full siblings. Even if no learning occurs, siblings can resemble each other because they share a common maternal environment, which may or may not have a heritable basis, and because siblings share non-additive, in addition to additive, genetic variation. Body size at birth and at weaning in mammals often exhibits strong maternal effects (Falconer 1981). However, because we controlled for age by restricting our analysis to 2- to 5-day-old pups and used forearm length as a covariate, similarities in vocalizations as a result of similar body sizes should have little influence on our estimates of genetic parameters. Although we have no way of estimating the magnitude of non-additive genetic factors, it is worth remembering that the covariance among full

siblings is composed of one-half the additive genetic variation but only one-quarter of the dominance variation and one-eighth of the epistatic variation. Furthermore, being unable to distinguish between additive and non-additive genetic variation does not weaken a conclusion for a genetic contribution to isolation-call variation. However, because adult females share only additive genetic variation with their offspring, additive genetic variation must be present for the signature system to evolve or for the female recognition system to co-evolve with the signature system. Better estimates of the genetic contribution to call variation must await future half-sibling or cross-fostering studies.

ACKNOWLEDGMENTS

We thank F. Mayer, S. Steele and L. Wolfenbarger for assistance in the field, E. Gould and R. Dooling for loaning sound recording and analysis equipment, and D. O'Reilly, L. Langbehn and L. Slone for help in measuring calls and feeding captive bats. The Hutton, Easton, Busby and McClain families provided access to bat colonies and P. Trail assisted in obtaining captive animals. D. Carr, L. Douglass, D. Denman and E. Russek-Cohen provided statistical advice. We are grateful to E. Gould, E. Russek-Cohen and D. Yager for valuable comments and suggestions on the manuscript. The University of Maryland Animal Care and Use Committee reviewed and approved the captive bat work (protocol number R-90-21). This work was supported by a Searle Scholar Award from the Chicago Community Trust to G.S.W. and the University of Maryland computer science center.

REFERENCES

Balcombe, J. P. 1990. Vocal recognition of pups by mother Mexican free-tailed bats, *Tadarida brasiliensis mexicana*. *Anim. Behav.*, **39**, 960–966.

Balcombe, J. P. & McCracken, G. F. 1992. Vocal recognition in Mexican free-tailed bats: do pups recognize mothers? *Anim. Behav.*, **43**, 79–88.

Barclay, R., Fenton, M. B. & Thomas, D. 1979. Social behaviors of the little brown bat, *Myotis lucifugus*. II. Vocal communication. *Behav. Ecol. Sociobiol.*, **6**, 137–183.

Becker, W. A. 1975. *Manual of Quantitative Genetics*. 3rd edn. Pullman: Washington State University Press.

Beecher, M. D. 1982. Signature systems and kin recognition. *Am. Zool.*, **22**, 477–490.

Boake, C. R. B. 1989. Repeatability: its role in evolutionary studies of mating behavior. *Evol. Ecol.*, **3**, 173–182.

Brown, P. 1976. Vocal communication in the pallid bat, *Antrozous pallidus*. *Z. Tierpsychol.*, **41**, 34–54.

Brown, P., Brown, T. & Grinnell, A. 1983. Echolocation, development, and vocal communication in the lesser bulldog bat, *Noctilio albiventris*. *Behav. Ecol. Sociobiol.*, **13**, 287–298.

Brown, P., Grinnell, A. & Harrison, J. B. 1978. The development of hearing in the pallid bat, *Antrozous pallidus*. *J. comp. Physiol.*, **126**, 169–182.

Casseday, J. H. & Covey, E. 1992. Frequency tuning properties of neurons in the inferior colliculus of an FM bat. *J. comp. Neurol.*, **319**, 34–50.

Esser, K.-H. & Schmidt, U. 1989. Mother–infant communication in the lesser spear-nosed bat *Phyllostomus discolor* (Chiroptera: Phyllostomidae): evidence for acoustic learning. *Ethology*, **82**, 156–168.

Esser, K.-H. & Schmidt, U. 1990. Behavioral auditory thresholds in neonate lesser spear-nosed bats, *Phyllostomus discolor*. *Naturwissenschaften*, **77**, 292–294.

Falconer, D. S. 1981. *Introduction to Quantitative Genetics*. 2nd edn. New York: Longman.

Fenton, M. B. 1985. *Communication in the Chiroptera*. Bloomington: Indiana University Press.

Gelfand, D. L. & McCracken, G. F. 1986. Individual variation in the isolation calls of Mexican free-tailed bat pups (*Tadarida brasiliensis mexicana*). *Anim. Behav.*, **34**, 1078–1086.

Grafen, A. 1990. Do animals really recognize kin? *Anim. Behav.*, **39**, 42–54.

Gould, E. 1971. Studies of maternal–infant communication and development of vocalizations in the bats *Myotis* and *Eptesicus*. *Comm. Behav. Biol.*, **5**, 263–313.

Gould, E. 1975. Experimental studies of the ontogeny of ultrasonic vocalizations in bats. *Devl Psychobiol.*, **8**, 333–346.

Gustin, K. & McCracken, G. F. 1987. Scent recognition in the Mexican free-tailed bat, *Tadarida brasiliensis mexicana*. *Anim. Behav.*, **35**, 13–19.

Hepper, P. G. 1986. Kin recognition: functions and mechanisms. A review. *Biol. Rev.*, **61**, 63–93.

Johnson, R. A. & Wichern, D. W. 1988. *Applied Multivariate Statistical Analysis*. 2nd edn. Englewood Cliffs, New Jersey: Prentice–Hall.

Jones, C. 1967. Growth, development, and wing loading in the evening bat, *Nycticeius humeralis* (Rafinesque). *J. Mammal.*, **48**, 1–19.

Jones, G., Hughes, P. M. & Rayner, J. M. V. 1991. The development of vocalizations in *Pipistrellus pipistrellus* (Chiroptera: Vespertilionidae) during post-natal growth and the maintenance of vocal signatures. *J. Zool., Lond.*, **225**, 71–84.

Jouventin, P. 1982. *Visual and Vocal Signals in Penguins, their Evolution and Adaptive Characters*. Berlin: Verlag Paul Parey.

Lessells, C. M. & Boag, P. T. 1987. Unrepeatable repeatabilities: a common mistake. *Auk*, **104**, 116–121.

McCracken, G. F. 1984. Communal nursing in Mexican free-tailed bat maternity colonies. *Science*, **233**, 1090–1091.

Marler, P. 1959. Developments in the study of animal communication. In: *Darwin's Biological Work: Some Aspects Reconsidered* (Ed. by P. R. Bell), pp. 150–206. Cambridge: Cambridge University Press.

Matsumura, S. 1979. Mother–infant communication in a horseshoe bat (*Rhinolophus ferrumequinum nippon*): *development of vocalization. J. Mammal.*, **60,** 76–84.

Matsumura, S. 1981. Mother–infant communication in a horseshoe bat (*Rhinolophus ferrumequinum nippon*): vocal communication in three-week-old infants. *J. Mammal.*, **62,** 20–28.

Milliken, G. A. & Johnson, D. E. 1984. *Analysis of Messy Data. Vol. I: Designed Experiments.* New York: Van Nostrand Reinhold.

Moss, C. F. 1988. Ontogeny of vocal signals in the big brown bat, *Eptesicus fuscus.* In: *Animal Sonar Processes and Performances* (Ed. by P. E. Nachtigall & P. W. B. Moore), pp. 115–120. New York: Plenum Press.

Reubsamen, R. 1988. Ontogeny of the echolocation system in rhinolophid CF-FM bats: audition and vocalization in early postnatal development. In: *Animal Sonar: Processes and Performance* (Ed. by P. E. Nachtigall & P. W. B. Moore), pp. 335–340. New York: Plenum Press.

Rother, G. & Schmidt, U. 1985. Die Ontogenetische Entwicklung der Vokalisation bei *Phyllostomus discolor* (Chiroptera). *Z. Saugetierk.*, **50,** 17–26.

SAS Institute. 1989. *SAS/STAT User's Guide, Version 6.06.* Cary, North Carolina: SAS Institute.

Sayigh, L. S., Tyrack, P. L., Wells, R. S. & Scott, M. D. 1990. Signature whistles of free-ranging bottlenose dolphins *Tursiops truncatus*: stability and mother–offspring comparisons. *Behav. Ecol. Sociobiol.*, **26,** 247–260.

Schmidt, U., Joermann, G. & Schmidt, C. 1982. Struktur und Variabilitat der Verlassenheitslaute juveniler Vampirfliedermause (*Desmodus rotundus*). *Z. Saugetierk.*, **47,** 143–149.

Simmons, J. A. 1973. The resolution of target range by echolocating bats. *J. acoust. Soc. Am.*, **54,** 157–173.

Simmons, J. A., Fenton, M. B., Ferguson, W. R., Jutting, M. & Palin, J. 1979. Apparatus for research on animal signals. *Life Sci. Misc. Publ. Roy. Ont. Mus.*,

Simmons, J. A. & Grinnell, A. D. 1988. The performance of echolocation: acoustic images perceived by echolocating bats. In: *Animal Sonar: Processes and Performance* (Ed. by P. E. Nachtigall & P. W. B. Moore), pp. 353–385. New York: Plenum Press.

Simmons, J. A., Howell, D. H. & Suga, N. 1975. Information content of bat sonar echoes. *Am. Scient.*, **63,** 204–215.

Simmons, J. A. & Vernon, J. A. 1971. Echolocation: discrimination of targets by the bat, *Eptesicus fuscus. J. exp. Zool.*, **176,** 315–328.

Sokal, R. R. & Rohlf, F. J. 1981. *Biometry.* 2nd edn. New York: W. H. Freeman.

Thomson, C. E., Fenton, M. B. & Barclay, R. M. R. 1985. The role of infant isolation calls in mother–infant reunions in the little brown bat, *Myotis lucifugus* (Chiroptera, Vespertilionidae). *Can. J. Zool.*, **63,** 1982–1988.

Watkins, L. 1970. Observations on the distribution and natural history of the evening bat (*Nycticeius humeralis*) in northwestern Missouri and adjacent Iowa. *Trans. Kans. Acad. Sci.*, **72,** 330–336.

Watkins, L. & Shump, K. A., Jr. 1981. Behavior of the evening bat *Nycticeius humeralis* at a nursery roost. *Am. Midl. Nat.*, **105,** 258–268.

Wilkinson, G. S. 1992. Communal nursing in the evening bat, *Nycticeius humeralis. Behav. Ecol. Sociobiol.*, **31,** 225–235.

Wilkinson, G. S. & Chapman, A. S. 1991. Length and sequence variation in evening bat d-loop mtDNA. *Genetics*, **128,** 607–617.

Wilson, D. E. 1988. Maintaining bats for captive studies. In: *Ecological and Behavioral Methods for the Study of Bats* (Ed. by T. H. Kunz), pp. 247–263. Washington, D.C.: Smithsonian Institution Press.

Woolf, N. K. 1973. The ontogeny of bat sonar sounds: with special emphasis on sensory deprivation. Ph.D. thesis, Johns Hopkins University, Baltimore, Maryland.