

Supplementary Information for

**Pup directed vocalizations of adult females and males
in a vocal learning bat**

Ahana Aurora Fernandez^{1,2*} and Mirjam Knörnschild^{1,2,3}

¹ Animal Behavior Laboratory, Free University of Berlin, Berlin, Germany

² Museum für Naturkunde – Leibniz Institute for Evolution and Biodiversity Science, Berlin, Germany

³Smithsonian Tropical Research Institute, Balboa, Ancón, Panama

*** Correspondence:**

Ahana Aurora Fernandez

aa.fernandez@fu-berlin.de

Table of Contents:

Supporting results

Acoustic differences between pup-directed and adult-directed female vocalizations p. 2

 Table S1: Statistics of the multivariate GLM 1 p. 2

 Table S2: Statistics of the multivariate GLM 2 p. 2

 Results of the paired Wilcoxon-Test p. 2

No individual signature in MDs p. 3

 Table S3: Individual classification success of MDs by a DFA p. 3

 Table S4: Relation between MD sequences and pup vocalizations p. 4

Acoustic similarity between pups and males separated by population p. 4

 Fig. S1: Euclidean distances between pups and males separated by population p. 4

 Short discussion about acoustic similarity between males and pups p. 5

Supporting methods pp. 5-8

Study species p. 5

Social vocalizations and sound recordings pp. 5-6

Acoustic analyses pp. 6-8

 Individual signature in maternal directive calls p. 6

 Correlation between MD sequences and pups' vocal practice p. 7

 Isolation calls pp. 7-8

 Assessment of acoustic similarity between ICs of adult males and pups p. 8

References p. 8

Results

Acoustic differences between pup-directed and adult-directed female vocalizations

GLM 1: The first multivariate GLM included all measured acoustic features, namely LFCCs 1-20, peak frequency, minimum and maximum frequency as dependent variables, as well as call type, ID and ID*call type as independent variables.

Table S1. Statistics of the multivariate GLM 1.

| Independent variables | Wilks-Lambda | F-value (df) | η^2 | Significance |
|-----------------------|--------------|-------------------|----------|--------------|
| ID | 0.004 | 1.483 (120, 88.4) | 0.66 | 0.026 |
| call type | 0.87 | 7.466 (24, 17) | 0.91 | > 0.001 |
| ID*call type | 0.005 | 1.469 (120, 88.4) | 0.66 | 0.029 |

GLM2: The second GLM included the dependent variables which had no overlapping estimated marginal means for ID and call type (no interaction between ID and call type), i.e. only variables in which the acoustic changes between call types were consistent across all individuals.

Table S2. Statistics of the multivariate GLM 2.

| Independent variable | Wilks-Lambda | F-value (df) | η^2 | Significance |
|----------------------|--------------|-------------------|----------|--------------|
| ID | 0.424 | 0.939 (35, 145.5) | 0.15 | 0.57 |
| call type | 0.333 | 9.732 (7, 34) | 0.67 | > 0.001 |
| ID*call type | 0.316 | 1.308 (35, 145.5) | 0.20 | 0.13 |

Additional Paired Wilcoxon tests: Each dependent variable differed significantly between call types (same statistics for each variable: $V=21$, $p = 0.031$). Since our sample size was restricted to six individuals the p-value cannot drop below 0.031.

No individual signature in MDs

Although the overall classification success (25%) of the DFA was higher than expected by chance (7.7%), most MD sequences were not correctly classified to the respective female. The obtained classification success for each female was thus not significantly different from chance success (paired Wilcoxon test; $Z = -1.732$, $p = 0.083$).

Table S3. Individual classification success of MDs by a DFA.

| ID | Colony | Classification success | No of females in social group |
|--------|--------|------------------------|-------------------------------|
| F2_2 | SL1 | 42.9% | 2 (1) |
| F4_4 | SL2 | 50% | 5 (4) |
| F7_7 | E3 | 30% | 9 (8) |
| F8_8 | E3 | 0% | 9 (8) |
| F9_9 | F1 | 50% | 8 |
| F10_10 | F1 | 0% | 8 |
| F11_11 | B | 0% | 4 |
| F12_12 | B | 20% | 4 |
| F14_14 | TO | 66.7% | 3(2) |
| F15_15 | TO | 0% | 3(2) |
| F17_17 | TR | 0% | 2 |
| F19_19 | TR | 0% | 3 |
| F20_20 | TR | 20% | 3 |

Table S3: Individual classification success of the MD sequences is shown in column 3 (in %). The last column delineates the number of females present in the colony, numbers in brackets indicate a change in number of present females since some females disappeared over the course of the study.

No relation between MD sequences and pup vocalizations

A non-parametric Friedman test was conducted to analyze the temporal relation between MD sequences and different pup vocalization categories. There was no difference between the pup vocalization categories (Table S4, column 1) and their temporal occurrence (i.e. before, during, after MD production, Table S4, columns 2-4) ($\chi^2 = 5.33$, $df=2$, $p=0.07$). Furthermore, we conducted a chi-square test to analyze if the frequency of pup vocalization categories (Table S4, column 1) differed depending on whether they were emitted before (column 2) or after (column 4) MD sequences. The results showed that this was not the case ($\chi^2=0.131$, $p=0.9$).

Table S4. Relation between MD sequences and pup vocalizations

| Pup vocalization categories | Before (5 sec) | MDs | |
|-----------------------------|----------------|--------|---------------|
| | | During | After (5 sec) |
| IC | 27 % | 10 % | 24 % |
| PP | 26 % | 6 % | 19 % |
| CS | 4 % | 1 % | 3 % |
| TS | 17 % | 5 % | 14 % |
| EC | 17 % | 5 % | 13 % |
| S | 9 % | 73 % | 27 % |

Table S4: IC=isolation call, PP=pup proto syllable train, CS=syllable train with adult-like courtship song syllables, TS=syllable train with adult-like territorial song syllables, EC=echolocation calls, S=silence.

Acoustic similarity between males and pups separated by population

Because population affiliation could possibly influence the acoustic similarity between pups and males, we additionally calculated the Euclidean distances between males and pups separated by population. In six out of eight pups of the Curú (Costa Rica) population the Euclidean distances for their ICs to the ICs of the male from the same colony was smaller than to the mean value for the ICs of the males from the other colonies (Figure S1 A). In four out of six pups of the Gamboa (Panamá) population the Euclidean distances for their ICs to the ICs of the male from the same colony was smaller than to the mean value for the ICs of the males from the other colonies (Figure S1 B). The paired Wilcoxon Test was not significant but showed a trend ($V=78$, $p=0.1$).

Figure S1. Euclidean distances between pups and males separated by population.

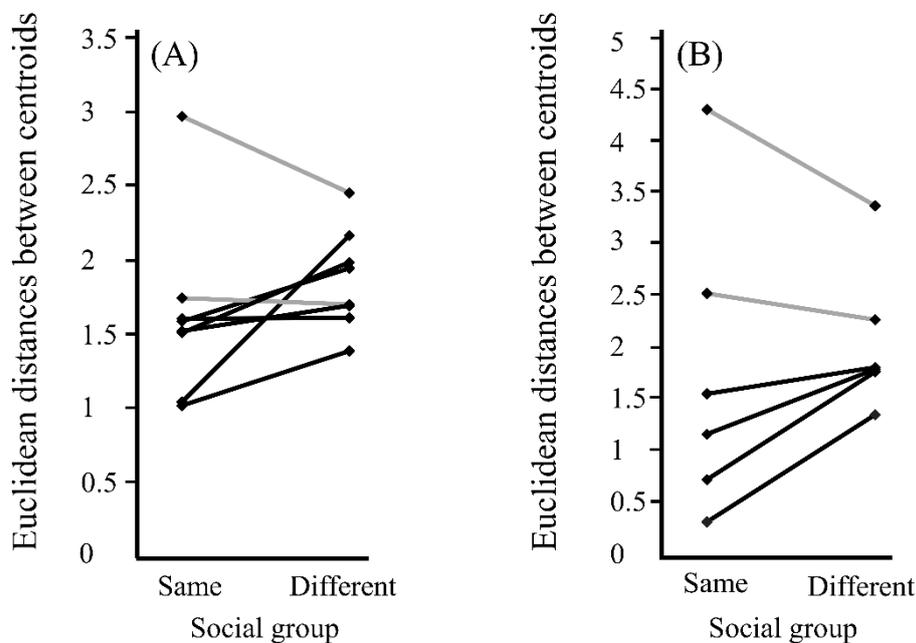


Figure S1. (A) Curú, Costa Rica: The Euclidean distance (i.e. proxy for acoustic similarity) between isolation calls of pups and males from the same social group is smaller than the distance between isolation calls of pups and males from a different social group in six out of eight pups. The data includes calls from 4 males, eight pups belonging to three different

colonies. (B) Gamboa, Panamá: The Euclidean distance (i.e. proxy for acoustic similarity) between isolation calls of pups and males from the same social group is smaller than the distance between isolation calls of pups and males from a different social group in four out of six pups. The data includes calls from 4 males, six pups belonging to three different colonies.

Short discussion about acoustic similarity between males and pups

In both populations, ICs of most pups had a smaller Euclidean distances to the ICs of males from the same colony than to the mean value for the ICs of males from the other colonies. This shows that there are acoustic differences between the two populations which have a certain influence on the acoustic parameters of the isolation call. However, our results show that despite the very small sample size and control for population influences in most of the juveniles, the acoustic similarity to the male of the own colony is still greater than to the males of the other colonies. Therefore, we believe that population influences play a rather minor role and our preliminary conclusions and conclusions drawn in this study are still valid. We believe that if the same analysis is performed with an adequate sample size (number of individuals per colony and number of calls per individual), these population influences are likely to be negligible.

Methods

Study species

Adult bats were individually identified by colored plastic rings on their forearms (A.C. Hughes Ltd. UK, size XCL; one band per forearm). Pups were first identified via their banded mothers, since mothers only nurse their own pups and ignore solicitation attempts of alien pups. At an age of approximately 60 days, when pups had almost adult mass and size, they were caught with mist nets (Ultrathin Mist Nets M-14; Ecotone, Gdynia, Poland) in the vicinity of the day-roosts, sexed and banded with individually distinct colored bands. Immediately after the procedure pups were released at the site of capture. The banding method is well established and did not cause any noticeable negative effect on the bats' behavior or welfare. It was not possible to catch all the pups because we wanted to reduce the disturbance at the day-roosts to a minimum and thus did not perform re-capture attempts. Therefore, some pups remained unsexed and unbanded (but, nevertheless, individual identification was possible via their banded mothers).

Social vocalizations and sound recordings

The majority of social interactions of this species take place during daytime, in the day-roost (Tannenbaum 1975; Behr and von Helversen 2004; Knörnschild et al. 2006; 2010). This species exhibits a large vocal repertoire (i.e. 25 distinct syllable types, Behr & von Helversen 2004; Knörnschild and von Helversen 2008; Thesis AA. Fernandez). Adult males are the vocally more active sex, they produce the majority of all syllables of the adult repertoire and produce two song types, the territorial and courtship song which are displayed on a daily basis in the day-roost (Behr and von Helversen 2004). Other vocalizations directed at adults include short calls during affiliative or agonistic interactions (Behr and von Helversen 2004). During ontogeny male pup-directed vocalizations are restricted to the isolation call (IC). During our study period, the male IC only occurred after a pup emitted an isolation call, never in relation to another vocalization type. The adult male IC production in response to pup IC was not specific; a male emitted IC in response to various other pups and vice versa, a pup IC could elicit ICs from various males. Females are much less vocal than males, in the day-roost they only produce the following vocalization types: maternal directives (MDs), echolocation calls (EC), during agonistic encounters screeches and noise bursts, in neutral context short variable syllables, inverted V-syllables, short and long chatter. Screeches, short variable syllables, echolocation calls and chatter are the most frequently produced vocalization types whereas inverted V-

syllables are emitted very rarely. MDs are the only pup-directed female vocalization and they are always only directed to the mother's own pup.

Pups acquire the adult vocal repertoire through a conspicuous pup vocal practice behavior during ontogeny (Knörnschild et al. 2006), a part of it through vocal imitation (Knörnschild et al. 2010). This vocal practice behavior is defined by daily vocal practice bouts which are reminiscent of infant canonical babbling (Knörnschild et al. 2006). During the first two weeks after pup birth, sound recordings were restricted to the main vocal activity period of *S. bilineata*, one hour at dawn and dusk. Pups attached to the mothers' belly exclusively produced several isolation calls (i.e. isolation call bouts) when being awake. After the first two weeks when pups were more active and detached from their mothers, acoustic recording sessions were extended to entire mornings (between 6am – 1 pm) and afternoons (1pm - 6pm) where focal pups were recorded *ad libitum*. At this point in time pups started to engage in vocal practice bouts. For each colony, we alternated between morning and afternoon sessions to avoid potential daytime biases in the vocal activity of pups. During these sessions we also recorded *ad libitum* adult vocalizations directed at pups.

Acoustic Analyses

Individual signature in maternal directive calls

Prior to acoustic measurements each sound file was bandpass filtered (5-120kHz) and normalized to 100% (Cool Edit 2000 Inc., Syntrillium Software Corporation P. O. Box 62255, Phoenix, AZ, USA). We used the software Avisoft SASLab Pro (v.5.2.09; R. Specht, Avisoft Bioacoustics, Glienicke, Germany) to measure acoustic parameters of the maternal directive calls (MDs) to test for an individual signature. Start and end of MDs within the sequence were determined automatically (-20dB relative to maximum amplitude, hold time 15ms). Automatic call detection was possible since the recordings had an excellent signal-to-noise ratio and MDs are monosyllabic. Subsequent to automatic call detection, acoustic parameters were measured automatically. Spectrograms were created using a Hamming window with 1024-point fast Fourier transform and 93.75% overlap (resulting in a 488 Hz frequency resolution and a time resolution of 0.128 ms). We restricted the acoustic parameter measurements to the fundamental frequency because it contained most of the sound energy. To reduce the temporal dependence of successive MDs within a sequence we subsequently averaged the acoustic parameter measurements over the entire sequence. For each MD we measured several temporal (duration, interval between syllables, time to maximum amplitude), and spectral parameters (minimum, maximum and peak frequency, bandwidth, entropy, harmonics-to-noise ratio) over the entire syllable. To estimate the frequency and entropy curvature of MDs we measured six spectral parameters (minimum, maximum and peak frequency, bandwidth, entropy, harmonic-to-noise ratio) at eleven different locations distributed evenly over the fundamental frequency of each syllable. Curvature parameters were obtained by performing principal component analyses (PCAs) with varimax rotation on the above mentioned parameters (one PCA on all 44 frequency parameters and another PCA on all 22 entropy and harmonic-to-noise ratio parameters), thus reducing multicollinearity between original acoustic parameters considerably. For the frequency curvature, we extracted six principal components (with eigenvalues > 1) which explained 95.5% of the total variance and for the entropy curvature five principal components (with eigenvalues > 1) which explained 71.1% of the total variance. Both PCAs fulfilled KMO and Bartlett's test criteria, thus assuring the appropriateness of our data for PCAs. We used the five most important derived acoustic parameters (three parameters describing the frequency curvature and two parameters describing the entropy curvature) per MD for subsequent statistical analyses.

Correlation between MD sequences and pups' vocal practice

MDs not only occurred during mother-pup reunions but also while the pup engaged in vocal practice behavior. Therefore, we investigated if MD production was related to a specific pup vocalization produced during vocal practice. Vocal practice in pups is reminiscent of canonical babbling in infants (Knörnschild et al. 2006; Fernandez 2020). During vocal practice bouts pups produce long syllable trains composed of undifferentiated proto-syllables only produced by pups and adult-like syllable types. The adult-like syllable types of those bouts can be classified into different syllable type categories.

We analyzed 134 MD sequences (N= 13 females). The five preceding and succeeding seconds of each MD sequence were scanned to investigate if MDs were produced after a specific pup vocalization (i.e. in the preceding five seconds) or if MDs elicited the production of a specific pup vocalization (i.e. potential pup vocalizations emitted simultaneously to MDs and the succeeding five seconds). Six different categories were established: Echolocation call (EC), isolation call (IC), pup proto-syllables (PP: syllable train mainly composed of proto-syllables), syllable train containing courtship song syllables (CS), syllable train containing territorial song syllables (TS) and silence (S) if the pup did not vocalize.

Isolation calls

Prior to acoustic measurements, all sound files were bandpass filtered (5-120 kHz) and normalized to 100% (Cool Edit 2000 Inc., Syntrillium Software Corporation P. O. Box 62255, Phoenix, AZ, USA). For the acoustic parameter measurements we used the software Avisoft SasLab Pro. The start and end of the syllables were defined manually, based on the oscillogram, and subsequently, automatic parameter measurements provided by the software were applied. Spectrograms were created using a Hamming window with 1024-point fast Fourier transform and 87.5% overlap resulting in a frequency resolution of 488 Hz and a temporal resolution of 0.256 ms. Although isolation calls are multiharmonic, we used only the fundamental frequency for automated parameter measurements because it contained most of the sound energy. The multisyllabic isolation call is composed of simple tonal calls (variable simple syllable: VS) succeeded by the end syllables (ES). The end syllable is composed of two syllable types, the composite (cs) and the simple stereotyped syllable (ss; following Knörnschild and von Helversen, 2008). The composite syllable is constituted of a facultative noisy part (nc) followed by a tonal (tc) part. For each syllable type or syllable part we measured several temporal (duration, interval between syllables, time to maximum amplitude) and spectral (minimum, maximum and peak frequency, bandwidth, entropy, harmonics-to-noise ratio) parameters. Entropy is a measure of the width and uniformity of the power spectrum and assesses how tonal or noisy a signal is (on a scale of 0-1, pure tone=0, white noise=1). We decided to not include the measurements of the noisy part of composite syllables in statistical analyses because this part was not always present in all end syllables. To estimate the entropy and frequency curvatures of end syllables, we measured the spectral parameters at seven different locations evenly distributed over the fundamental frequency of each syllable type and part. We obtained the curvature parameters by performing a principal component analyses (PCAs) with varimax rotation on the aforementioned spectral parameters (one PCA on all 28 frequency parameters and another on all 14 entropy and harmonics to noise ratio parameters). For the frequency curvature we extracted four principal components (with eigenvalues >1) which explained 94.8% of the total variance for tc and five principal components (with eigenvalues >1) which explained 94.7% of the total variance for ss. For the entropy and harmonic-to-noise ratio (hnr) curvature, we extracted four principal components (with eigenvalues > 1) which explained 73.5% of the total variance for tc and three principal components (with eigenvalues >1) which explained 69.5% of the total variance for ss. All PCAs fulfilled Kaiser-Meyer-Olkin (KMO)

and Bartlett's criteria, thus assuring the appropriateness of our dataset for PCAs. Hence, we obtained 16 derived acoustic parameters per isolation call for subsequent statistical analyses.

Assessment of acoustic similarity between ICs of adult males and pups

To obtain the centroids of each individual (i.e. the position of each individual in acoustic space) we performed a discriminant function analysis (DFA). We adjusted the DFA to the unequal number of analyzed calls per individual by computing group sizes based on prior probabilities. The number of variables introduced into a DFA should be lower than the smallest number of cases per group to avoid statistical overfitting. In our case, this was not possible since we sometimes only had 4 cases per group (i.e. 4 IC per individual). We selected 12 acoustic parameters (LFCC1, frequency curvatures 1-4 of the tc part, frequency curvatures 1-2 of the ss syllable, the entropy-hnr curvatures 1-2 of the ss syllable, the entropy-hnr curvature 1 of the tc part of the composite syllable, mean maximal frequency of the tc part of the composite syllable and the ss syllable) to obtain the centroids for a subsequent assessment of acoustic similarity. All parameters were checked for multicollinearity and introduced simultaneously into the DFA. We obtained a multidimensional space defined by four discriminant functions in which group centroids marked the position of each individual in the DFA signal space. Subsequently, we calculated Euclidean distances between centroids of pups and males from the same or different social groups.

In addition, based on the originally calculated centroids, we calculated the Euclidean distances between pups and males of the same or different colony separately for each population (Costa Rica and Panamá).

References

- Behr, O., & von Helversen, O. (2004). Bat serenades—complex courtship songs of the sac-winged bat (*Saccopteryx bilineata*). *Behav. Ecol. Sociobiol.*, 56(2), 106-115. doi: 10.1007/s00265-004-0768-7
- Fernandez, A. A. (2020) Vocal ontogenetic processes in bat pups. From babbling behavior to the interplay of social and vocal complexity. [dissertation]. [Berlin (GE)]: Free University of Berlin, Germany.
- Knörnschild, M., Behr, O., and von Helversen, O. (2006). Babbling behavior in the sac-winged bat (*Saccopteryx bilineata*). *Naturwissenschaften* 93, 451-454. doi: 10.1007/s00114-006-0127-9.
- Knörnschild, M., and von Helversen, O. (2008). Nonmutual vocal mother–pup recognition in the greater sac-winged bat. *Anim. Behav.* 76, 1001-1009. doi: 10.1016/j.anbehav.2008.05.018.
- Knörnschild, M., Nagy, M., Metz, M., Mayer, F., and von Helversen, O. (2010). Complex vocal imitation during ontogeny in a bat. *Biol. Lett.* 6, 156-159. doi: 10.1098/rsbl.2009.0685.