Effects of deafening on vocal production learning in the Egyptian fruit-bat

Julie E. Elie[1*], Sandra E. Muroy[2,3], Daria Genzel[1], Tong Na[4], Lisa A. Beyer[5], Donald L. Swiderski[5], Yehoash Raphael[5], Michael M. Yartsev[1,6*]

[1]Department of Bioengineering, University of California, Berkeley; Berkeley, CA 94708, USA.

[2]Department of Molecular and Cell Biology, University of California, Berkeley; Berkeley, CA 94708, USA.

[3]Department of Integrative Biology, University of California, Berkeley; Berkeley, CA 94708, USA.

[4]Department of Electrical Engineering and Computer Sciences, University of California, Berkeley; Berkeley, CA 94708, USA.

[5]Department of Otolaryngology–Head and Neck Surgery, Michigan Medicine, Kresge Hearing Research Institute; University of Michigan, 1150 Medical Center Drive, Ann Arbor, MI, 48109-5616, USA.

[6]Helen Wills Neuroscience Institute, University of California, Berkeley; Berkeley, CA 94708, USA.

* Corresponding authors: Julie E Elie, julie.elie@berkeley.edu; Michael M Yartsev, myartsev@berkeley.edu

Abstract

Some species have evolved the ability to use the sense of hearing to modify existing vocalizations, or even create new ones. This ability corresponds to various forms of vocal production learning that are all possessed by humans, and independently displayed by distantly related species. Among mammals, a few species, including the Egyptian fruit-bat, would possess such vocal production learning abilities. Yet the necessity of an intact auditory system for the development of the Egyptian fruit-bat typical vocal repertoire has not been tested. Here we addressed this gap by eliminating pups' sense of hearing at birth and assessing its effects on vocal production in adulthood. The deafening treatment enabled us to both causally test these bats vocal learning ability and discern learned from innate aspects of their vocalizations. Leveraging wireless individual audio recordings from freely interacting adults, we show that a subset of the Egyptian fruit-bat vocal repertoire necessitates auditory feedback. Intriguingly, these affected vocalizations belong to different acoustic groups in the vocal repertoire of males and females. These findings open the possibilities for targeted studies of the mammalian neural circuits that enable sexually dimorphic forms of vocal learning.

Significance

Vocal production learning is the rare capacity amongst animals where hearing is used to modify or create new vocalizations. The Egyptian fruit-bat is believed to possess this capacity, yet whether they need audition to achieve a mature vocal repertoire is unknown. Furthermore, a systematic causal examination of learned and innate aspects of the entire repertoire has never been performed in a vocal learner. Here, we addressed these major gaps directly by abolishing hearing in Egyptian fruit-bats at birth and investigating its effects on the vocal production in adulthood. Leveraging simultaneous individual wireless audio-recordings from freely interacting

adult bats, we identify the subset of learned vocalizations and provide evidence that vocal learning is sexually dimorphic in that species.

Introduction

Vocalizations constitute the building blocks of the acoustic code used for vocal communication. The efficiency and complexity of a vocal communication system depends on the species ability to produce a diversity of such building blocks. While this diversity can be supported by hard wired motor programs that are innate (e.g., motor programs of male mouse song[1]), the sound diversity is also enhanced in some species by their ability to modify existing calls or create new ones[2–7]. This ability is defined as vocal production learning and has been examined in a wide variety of species[2,4,8]. Yet, experiments discerning innate versus learned vocalizations in the entire repertoire of a single species are missing.

The Egyptian fruit-bat has been proposed as one of the species capable of vocal production learning after several studies demonstrated how manipulation of either the social or physical environment affects the acoustic characteristics of their communication calls[9–11]. However, it remains unknown whether all or only a subset of their repertoire is acquired through learning. Furthermore, the acoustic characteristics of the voice of individuals can easily be mistaken for an effect of vocal learning when comparing the vocal behaviors of two groups of animals (one control and one experimental). Previous conclusions on vocal learning abilities are weakened by the lack of reliable identification of vocalizing individuals[9–11]. Combined, these gaps limit thorough exploration of the neural mechanisms of vocal production learning in this species, although cutting-edge technologies for measuring brain activity are readily available[12–15]

Here we reasoned that because vocal production learning heavily depends on the sense of hearing, the main sensory feedback that the nervous system can use to change or create

new motor programs[16], abolishing this feedback during development can begin revealing what aspects of the repertoire are learned and which are innate. Therefore, we conducted the first causal study of vocal production learning in the Egyptian fruit-bat by establishing a method for deafening pups shortly after birth and testing the impact of such manipulation on the vocal behavior of each of these bats when they became adults.

Results

Deafening at birth and recording of vocalizations at adulthood.

The acoustic isolation of newborn pups to any bat vocalization was ensured by single-housing pregnant mothers before pup birth in acoustically isolated chambers. Deafening of the pups was then chemically achieved shortly after birth (Figure 1A; n=10 bats). Five pups received an injection regimen of the deafening antibiotic kanamycin, while five pups received control saline injections (Methods). In each experimental group of five, there were three females and two males. The extent of hearing loss incurred was verified both functionally, using auditory brainstem recordings (Figure 1B, Supplementary Figure 1; Methods), and histologically, by examining the cochlear epithelium (Figure 1C, Supplementary Figure 1; Methods). These approaches revealed profound deafness in the kanamycin treated bats and normal hearing in the saline treated bats.

Because the ontogeny and maturation of the Egyptian fruit-bat vocal behavior is unknown, we focused our investigation of the vocalizations of hearing and deaf bats when they were fully mature adults (Figure 1A and 1D, Methods). By then, the bats had lived for years in a communal social environment where they were equally exposed to conspecific vocalizations. Over a seven week period, the deaf and hearing adults were co-housed and daily equipped with wireless audiologger collars, that enabled to detect all vocalizations from the bat repertoire and to unambiguously assign the vocalizer identity to each vocalization[13]. Additionally, an ambient

microphone further ensured high quality audio recordings of the vocalizations. After manual curation, we obtained thousands of calls from both hearing (n=16,937) and deaf bats (n=11,154).

No effect of deafening on the whole repertoire when controlling for identity.

Deafening did not affect the vocal activity of bats (Supplementary Figure 2) and we obtained a similar number of vocalizations from both deafened and hearing bats (>1,000 vocalizations on average per individual). To explore if deafening was affecting the acoustic features of vocalizations, we used both a classical bioacoustical approach (28 predefined acoustic features measured on each call) and a data driven approach (Figure 1D). In the latter approach, we used the Modulation Power Spectrum[17] (MPS) to describe the spectro-temporal modulations of vocalizations. The resulting MPS of each vocalization was used both in UMAP projections of all vocalizations and in regularized linear classifiers that quantified the level of discrimination between calls emitted by deaf and hearing bats (Regularized Permutation Discriminant Function Analysis, RPDFA, see Methods). Unexpectedly, with regards to previous reports[9–11], when we considered all the recorded vocalizations, none of the two approaches revealed any effect of deafness when the identity of the vocalizing bats was accounted for (Supplementary Figure 3, all 28 linear mixed-effects models on acoustic features with $p>0.05$). The performance of the RPDFA at classifying call MPS between deaf and hearing bats was 60.4% ± 1% and not significantly different from the chance level of classifying any random two groups of five bats, 62.4% ± 1.3% (Supplementary Figure 4A and 4B; note that considering the voice characteristics of individuals raises the chance level higher than 50%).

Heterogeneity in vocal space coverage according to sex and hearing ability.

116 However, we next considered the possibility that not all vocalizations necessarily had to
117 depend on auditory feedback. That is, some could be innate whereas others could be learned.
118 Therefore, considering all vocalizations together, as was done in previous studies, may result in
119 masking the effect of deafening. Indeed, linear measures of acoustic proximity revealed that
120 bats were not homogeneously occupying the vocal space (Figure 2). First, we found that males
121 covered a larger vocal space than females (vocalizations with large values of Sex Dissimilarity;
122 Figure 2A and Supplementary Figure 5A, 5B and 5D) and that for each sex, some vocalizations
123 were distinct between deaf and hearing bats (Supplementary figure 6A-6C; performance of the
124 RPDFA against identity controlled chance level: all male calls, 67.4 ± 1.2% vs 64.5 ± 0.8%; all
125 female calls, 62.0 ± 0.6% vs 58.6 ± 1.3%). Second, a small proportion of the repertoire of each
126 sex was idiosyncratic to the hearing ability of bats (Figure 2B and 2D). In males, these
127 distinctive vocalizations represented 23.9 ± 3.2% of hearing bats repertoire and could not be
128 matched with similar calls from deaf bats, indicating that hearing males produced vocalizations
129 that deaf males did not (Figure 2B, Supplementary Figure 7). In females, the few distinctive
130 vocalizations represented 4.5 ± 1.5% of deaf bats repertoire and did not have a match with
131 vocalizations produced by hearing females, indicating that these deaf females were producing a
132 few atypical vocalizations (Figure 2D, Supplementary Figure 7). These vocalizations belonged
133 to regions of the vocal space that grouped the loudest, more tonal, and higher pitch
134 vocalizations in the repertoire (Supplementary Figure 8).
135 To further quantify and identify the spectro-temporal differences between deaf and
136 hearing bats' vocalizations throughout the repertoire, we parsed the vocal space into four
137 groups (Figure 2C, Supplementary Figure 9). This exact number of groups optimized both
138 maximum division and cluster quality (Supplementary Figure 10). Deafness affected
139 vocalizations in two of the four groups (Supplementary Figure 4; performance of the RPDFA
140 against the identity-controlled chance level: Group 1, 73.2 ± 3.7% vs 66.8 ± 3.2%; Group 4, 85.9
141 ± 1.8% vs 76.9 ± 2.5%). Group 4 corresponded to loud and high pitch vocalizations that females

142 rarely produced (Figure 2A, Supplementary Figure 5C, 8 and 9) and only male calls were
143 different between hearing and deaf individuals in this acoustic group (Supplementary figure 6G-
144 6I; performance of the RPDFA against identity controlled chance level: Male Group 4 calls, 89.5
145 ± 2.7% vs 79.2 ± 2.1%; Female Group 4, 62 ±10.4% vs 59.7 ± 9.2%). Surprisingly, although
146 both sexes produced calls that belonged to acoustic Group 1 (Supplementary Figure 5C), only
147 female calls were distinct between hearing and deaf bats (Supplementary figure 6D-6F;
148 performance of the RPDFA against identity controlled chance level: Male Group 1 calls, 69.9 ±
149 5.4% vs 65.7 ± 3.9%; Female Group 1, 76.4 ± 1.9% vs 61.2 ± 3.1%). Combined, these findings
150 suggest that a subset of the Egyptian fruit bat repertoire depended on intact auditory feedback
151 in a sexually dimorphic manner.
152

153 Effect of deafening on the spectro-temporal patterns of a subset of male and 154 female calls.

155 The acoustic differences in the vocalizations of deaf and hearing bats were a complex
156 combination of both spectral and temporal parameters. The atypical vocalizations from deaf
157 females in acoustic group1 were characterized by complex patterns of pitch modulations and
158 decreased formants power (Supplementary figure 11). However, these differences were not
159 captured by the classical bioacoustical approach (Supplementary figure 12). In contrast, the
160 pattern of spectro-temporal modulations that characterized hearing male calls from acoustic
161 group 4 was clear. Hearing males produced simple downsweep calls that were never matched
162 by deaf males who produced calls with more upsweeps (Figure 3, Supplementary figure 13).
163 Furthermore, deaf male calls had higher spectral content (i.e. more harmonics) than hearing
164 male calls as measured by spectral features such as the third spectral quartile (Figure 3,
165 Supplementary figure 13).
166

Discussion

In this study, we assessed the necessity of hearing for the development of a normal vocal repertoire in Egyptian fruit bats. The acoustic analysis demonstrated that the repertoire of this species consists of a large majority of innate vocalizations (>75% for males and >95% for females). Auditory input is not necessary to establish, fine-tune or maintain the neural motor programs controlling the production of these calls. However, some vocal features were not produced by males that lacked auditory input (downsweep calls with filtered higher harmonics) and atypical spectro-temporal patterns emerged in a few calls from deaf females, indicating that neural motor programs for these calls might be plastic and require an intact auditory input to properly mature. According to the source-filter theory that was first developed to explain human voice production[18,19] and then applied to animal vocalizations[20–22], the upsweeps and downsweeps of the fundamental frequency are controlled by the vocal source (the larynx), while the filtering of call harmonics and formants are achieved by change in the shape of the vocal tract (e.g., vocalizing with open or closed mouth). Under this theory, the results of the present acoustic analyses support the view that male Egyptian fruit-bats need to hear themselves, and/or other bats, in order to properly control both their larynx and upper vocal tract to produce the downsweep calls. The hypothesis of a fine motor control of the larynx is consistent with our recent findings demonstrating a direct monosynaptic pathway from the vocal-motor cortex to the brainstem center innervating the larynx[12]. These later findings are, so far, the strongest evidence of the existence of this direct connection in mammals, which has been suggested as a central anatomical hallmark of the neural circuitry that enables vocal production learning[23–27].

Previous experiments, such as pups reared in social isolation, or manipulation of the acoustic environment of both young and adults, had shown effects when considering together all vocalizations in the Egyptian fruit bat repertoire[9–11]. Deafening would also affect all the vocalizations in the adult repertoire of the pale spear-nosed bat, *Phyllostomus discolor*[28]. We

192 cannot exclude that in our experiment, deaf bats could have compensated for their lack of
193 hearing by using somatosensation as a sensory feedback and social responses of their hearing
194 companions as guiding cues to produce most of the vocalizations in their repertoire.
195 Somatosensation is indeed a feedback that is used along with audition to guide vocal production
196 in humans and songbirds alike[29–31]. Nonetheless, even after years of communal living, males
197 could not properly produce a significant proportion of their vocal repertoire in the absence of
198 auditory feedback. These results pave the way to targeted studies of these male Egyptian fruit
199 bat vocalizations, with the possibility for comparing the neural mechanisms of production
200 between learned and innate vocalizations in a mammal brain. Furthermore, the observed
201 difference between males and females opens new opportunities to investigate how the sex of an
202 individual might interfere with the maturation of vocal behavior in a mammalian brain that is
203 particularly suitable for neurophysiological research.
204

205 # Methods

206 ## Subjects and housing

207 All experimental procedures were approved by the Institutional Animal Care and Use Committee
208 of the University of California, Berkeley. Nine wild-caught adult female Egyptian fruit-bats
209 (*Rousettus Aegyptiacus*) and their ten pups (five females and five males) were used for this
210 study. Each female gave birth to one pup except for one female that gave birth twice. The
211 progenitor female bats were imported from Israel and housed in our lab colony at the University
212 of California Berkeley for at least six months before being included in the experiments. Female
213 bats were isolated in soundproof booths when they were highly pregnant (individual cage size:
214 10x12x20 inches). This protocol has been previously used to ensure that pups would not be
215 exposed to any vocalization including their mother's vocalizations[10]. Indeed, an isolated mother
216 does not vocalize even in presence of its pup[10]. Each female was maintained with its pup in

217 their soundproof booth until the end of the deafening protocol (see below) of the pup. After the
218 end of the injection protocol (post-natal day 56), the young bats were moved to a collective
219 housing room and housed together with their mothers and male bats from the colony in larger
220 cages (21 x 32 x 35.8 inches). Each cage contained one saline pup and one kanamycin pup
221 with their respective mothers and two adult males. At sexual maturity (7 months old), the young
222 bats were separated from their mothers and housed in single sex cages with age matched
223 unmanipulated bats.
224 When reaching 3-4 years of age, all ten experimental bats were grouped together in a large
225 cage (180x60x60 cm) and isolated from the rest of the colony in a soundproof room for two
226 months over which their vocalizations were recorded as detailed below.
227 In all cages, bats were maintained on a reverse day-light cycle set at 12h/12h. Recordings of
228 vocalizations were conducted during the dark period.
229

230 ## Deafening protocol

231 Half of the pups followed a treatment by kanamycin (powder from Alfa Aesar diluted in PBS, pH
232 7.4, Gibco 10010-02B) while the other half were kept as control subjects and injected with saline
233 solution (PBS, pH 7.4, Gibco 10010-02B). Starting on post-natal day 32 (PND32) and for 21
234 days, the pups were subcutaneously injected twice daily (8 hrs apart). The dosage of kanamycin
235 was set to increase stepwise over the 21 days (6 days at 300 mg/kg, 5 days at 400 mg/kg, 5
236 days at 500 mg/kg and 5 days at 600 mg/kg). The weight of the young bats was measured at
237 most 4 days before a change of dosage or at least on the eve of a change of dosage to correctly
238 adjust dosage. Control subjects were injected with the same volume of saline solution as the
239 volume they would have been injected with if they were kanamycin subjects.

Audiogram

Eight months after the end of the acoustic recordings, the audiogram of each experimental bat was established by measuring their auditory brain response (ABR) to tone pips of 2.5 ms (Hann window) spanning the auditory range expected for that species (1.5 - 90kHz, 25 logarithmically spaced levels of frequencies) and presented at various levels of intensity (30-110 dB SPL, 9 steps of 10dB SPL), following the same protocol as Linnenschmidt and Wiegrebe (2019)[32]. Briefly, bats were isolated and fasted from 1h and up to 2h before inducing anesthesia by subcutaneous injection of a mix of Ketamine (22 mg/kg), DexaMedetomidine (0.09 mg/kg) and Midazolam (0.45 mg/kg). Once the bat was deeply anesthetized, it was placed laying on its stomach on a heating pad in a soundproof room, facing the loudspeaker (Scanspeak R2604/833000) at 5 cm, with an oxygen delivery tube placed right under its nose (flow rate ~300mL/min). The animal temperature and breathing rate were monitored every 15 min during the procedure. The ABR was measured as the difference of potential between a signal subdermal electrode inserted at the base of the head in the neck of the animal and a reference subdermal electrode inserted subcutaneously atop of the head between the two ears. The whole system was grounded by an alligator clip placed on the lower part of one ear. The electrode signals were amplified by a TDT headstage (RA4Li, Tucker Davis Technologies Inc., Fl, USA) and further amplified and digitized by an audio interface (Fireface UC, RME, Haimhausen, Germany; sampling rate 192 kHz). After bandpass filtering between 100 Hz and 3 kHz and downsampling 10 times, the electrophysiological signals were stored for offline analysis. The 225 (nine amplitude levels and 25 frequency steps) tone pips were each presented 256 times at a repetition rate of 44Hz. Using previously used matlab algorithm[32], tone pips were generated at 192kHz, digitally equalized in magnitude and phase to compensate for the loudspeaker impulse response, converted to analog using the same audio interface as the

265 one used for processing the ABR signal, and amplified using one channel of an audio amplifier
266 (AVR 347, Harman Kardon, Stamford, CT, USA).
267 For each of the 256 presentations of a tone pip, the ABR signal was extracted in two
268 windows of 7 ms, one aligned to the tone pips (response window) and the second one randomly
269 placed between 0 and 23ms after stimulus onset (background noise window). From these 256
270 pairs of snippets of signal, we generated one tone response (the average electrophysiological
271 signal over all response windows) and one background response (the average
272 electrophysiological signal over all background windows) and measured the RMS of each. We
273 bootstrap the calculation of the RMS background response for each stimulus by repeating 1000
274 times the selection of random background noise windows in the 256 ABR time series. The exact
275 p-value of obtaining a tone response RMS higher than the background response RMS values
276 was then calculated as the number of background response RMS values equal or higher than
277 the tone response RMS for that stimulus. We could then draw the audiogram for each bat as the
278 lowest sound amplitude, for each carrier frequency tested, at which the RMS of the ABR signal
279 was significant at p=0.05 (see Supplementary Figure 14).
280

281 ## Histology

282 *Cochlear extraction*

283 After ABR measurements, the bats still under anesthesia were euthanized by a lethal
284 intraperitoneal injection of sodium pentobarbital. The body was then transcardially perfused with
285 200mL of phosphate buffer saline (PBS) solution (pH=7.4) spiked with 0.5mL of Heparin (1,000
286 USP units/mL) followed by 250 mL of fixative (3.7% formaldehyde PBS solution, pH=7.4). The
287 temporal bones were removed from the skull and the inner ears isolated. The oval and round
288 window were opened to facilitate flow of fixative solution for rapid fixation. Inner ear tissues were
289 shipped to the University of Michigan for histological analyses.

290 The cochleae with their surrounding bone were decalcified in 5% EDTA until the bone was soft
291 to facilitate dissection of the sensory area, the organ of Corti. The tissues were rinsed in PBS
292 and further dissection was done to remove the bone, stria vascularis and tectorial membrane.
293
294 *Phalloidin staining*
295 The cochleae were permeabilized with 0.3% Triton X-100 in PBS for 15 minutes followed by
296 incubation with Alexa Fluor 488 phalloidin (Invitrogen A12379) for 40 minutes, all at room
297 temperature. After rinsing with PBS, the cochleae were further dissected into segments of the
298 basal turn of the cochlea (higher frequencies area) and the apical turn (lower frequencies).
299 Segments were then mounted on slides with Prolong Gold (Invitrogen P36930). The slides were
300 stored in the dark at 4°C until viewed with fluorescence microscopy.
301
302 *Imaging of the organ of Corti*
303 The samples were imaged with a Leica DMRB epifluorescence microscope. Images were
304 captured using a CCD cooled SPOT-RT monochrome digital camera. Digital images were
305 processed with Adobe Photoshop.
306
307 ## Audio recordings
308 When the animals reached 3-4 years old, the ten experimental bats were recorded for 35 days
309 over the course of seven weeks, for 5-7 hours per day during their night cycle. For acclimation
310 purposes, the bats were all housed in the recording cage starting 16 days before the first day of
311 audio recording and were housed in that recording cage for the entire duration of the recording
312 period (7 weeks). The cage was a rectangular prism (180 x 60 x 60 cm) that had two sides
313 made of plexiglass, thereby permitting clear remote visual monitoring of bats behavior via two
314 cameras (Flea 3 FLIR). The remaining sides of the enclosure were made of plastic mesh,

allowing bats to easily perch and crawl on the surfaces. The enclosure was placed in an acoustically shielded room and all recording sessions were conducted during the dark cycle under red LED light. All bats were equipped daily with audio data-loggers to record and identify their vocalizations. Water was given *ad libitum* and the daily diet supply was placed into the cage at the beginning of each recording session to increase the diversity of behavioral contexts. The audio recordings of each individual bat vocalizations were performed using both an audio data-logger and an ambient ultrasonic microphone (Earthworks, M50) centered 20 cm above the cage ceiling and connected to the main computer unit via an analog to digital convertor (MOTU, 896mk3). The audio collected from the ambient microphone was recorded throughout the session (sampling rate of 192 kHz) using an in-house Matlab GUI (VocOperant; https://github.com/julieelie/operant_bats). As previously described (Rose et al 2021) the audio data-logger consists in a single-axis, low mass, piezo-ceramic accelerometers (BU-27135, Knowles Electronics, sensitivity 0-10kHz) that is mounted on a flexible rubber necklace placed against the throat of the subject, in a way that does not restrict normal behavior but enables the detection of laryngeal vibrations produced during vocalizations. The signal of the accelerometer is recorded, digitized at a sampling rate of 50 Hz, and saved on removable SD cards with a wireless audio data logging device (“audiologgers”; Audio Logger AL1 or AL2, Deuteron Technologies) mounted on the necklace on the back of the subject. The audiologger and its lithium-polymer battery are encapsulated in a house-made 3D-printed plastic casing to prevent damage to the electronics. All audiologgers were controlled and synchronized by a single transceiver. The Egyptian fruit bats in our experiment weighed more than 110 gr and could fly with ease while equipped with their audiologger. The synchronization between the microphone recording and the transceiver controlling the audiologgers was achieved using transistor-transistor logic (TTL) pulses generated by an UltraSoundGate Player 216H (Avisoft Bioacoustics) and sent via coaxial cables. The experimenter could monitor the behavior of the animals in an ante-chamber via the video cameras and the ambient microphone.

Vocalizations detection and acoustic analysis

From all the days of recording, we sampled 13 days for acoustical analysis. These days were chosen such as to optimize their spread across the 8 weeks of recording and optimize the recording conditions (no audiologger breaking incident).

Vocalization detection

Acoustic data logged as voltage traces on the SD cards of the audiologgers were extracted into Matlab files and aligned across bats simultaneously recorded using a custom-made Matlab code (https://github.com/NeuroBatLab/LoggerDataProcessing/). Potential vocalizations were then detected and segmented from these piezo recordings using an in-house series of Matlab scripts (https://github.com/NeuroBatLab/SoundAnalysisBats). The whole process consisted in three major steps: 1) detection of sound events on audiologger signals, 2) automatic classification between vocalizations and noise, 3) manual curation of potential vocalizations. Supplementary Figure 15 presents a full flow chart of the approach taken for the detection of vocalizations in audiologger recordings.

1. Detection: To focus on the detection of vocalizations emitted by the bat wearing the collar, the signal of each audiologger was first band-passed between 1 and 5kHz. As previously described, this frequency range is not contaminated by airborne vocalizations from other bats standing close to the collar wearer (Rose et al, 2021). After determining a noise threshold from sections of silence during the recording session for that audiologger (three times the RMS of silence sections), potential vocalizations were detected by threshold crossing on the amplitude envelope (RMS, sampling frequency 1kHz) and any sound event above threshold for longer than 7ms was kept.

2. Automatic step of data sorting between actual vocalizations and noise: Sound events closer than 50ms were merged as a single sound sequence and a battery of 20 acoustic measurements (pre-defined acoustic features, PAFs) were applied on them (see Elie and Theunissen, 2016, for mathematical definitions): RMS, maximum amplitude, the five first momentum of the amplitude envelope taken as a distribution (mean, standard deviation, kurtosis, skewness, and entropy), the five first momentum of the frequency spectrum taken as a distribution, the three quartiles of the frequency spectrum, the mean pitch saliency, and four parameters that pertain to the sound as recorded from the ambient microphone (RMS, mean and maximum of the amplitude envelope, and maximum value of cross-correlation between the microphone and the audiologger signals). Potential vocalizations among the detected sound events were then identified using these 20 acoustic parameters as input to a support vector machine (SVM) trained on the data of two sessions manually sorted between vocalizations and noise by an expert (JEE). This data was obtained from a total of 100 min of recording of 8 bats (800 min of audiologger data) and contained 24,633 sound elements, of which 1,377 were vocalizations. The automatic sorting was set to be very conservative of vocalizations by setting the threshold on the posterior probability of a vocalization at 0.002, with the drawback of yielding many false discoveries (percentage of vocalizations correctly identified in 10-fold cross validation: 98.3%; percentage of noise miss-classified as vocalizations in 10-fold cross validation: 7.5%).

After the manual curation (see below) of a reasonable dataset (five days), an extra step of automatic detection could be added to the pipeline to further filter out sequences devoid of vocalization. We trained a neural network on the manual annotations obtained from these five days of recording (4,936 vocalizations). The network was implemented using the PyTorch library and the architecture was composed of ResNet-50 with a sigmoid output layer. For each 100ms non-overlapping window of the audiologger signal,

we bandpass filtered the signal between 100Hz and 5kHz, and calculated its spectrogram using *soundsig* from the BioSound library (https://github.com/theunissenlab/soundsig) with the following parameters: sampling rate=1kHz; frequency spacing=100Hz; dynamic range=50dB. The spectrogram was then colorized using the color scale in *soundsig*. The training label for each 100ms window was 1 if there existed a vocalization in that window and 0 otherwise. The 3-channel color spectrograms and corresponding binary labels were used as inputs for the network. To take into account the large imbalance between noise and vocalization elements in the dataset, each training batch was set to contain 5 noise elements for each vocalization element. We used a weighted binary cross-entropy loss function with penalty=7.5 to further address the imbalance within each training batch. 20 percent of the data was held out for validation. We randomly initialized the network weights and trained the network for 10 epochs using the Adam optimizer with learning rate=1e-3. A sound sequence on a given audiologger was considered as worthy of manual examination if the maximum of all posterior probability values (one value per 100ms window) was higher than 0.9. At this threshold of maximum posterior probability value, the trained neural network achieved high performance of correct classification of sequences containing vocalizations (98.6%) with low values of false discovery (5.4% of noise sequences detected as containing vocalizations) enabling to greatly speed up the vocalization discovery procedure. The data from 8 more days of recording were evaluated by the trained neural network.

3. Manual curation: to further eliminate noise and check the identity of the bat producing vocalizations, each potential vocalization was aurally and visually scrutinized by trained students and an expert (JEE) based on the inspection of the spectrograms of its signal as recorded from the ambient microphone and from the audiologgers of all the bats

(*manual annotation* in Supplementary figure 15). Vocal utterances had to have a minimum duration of 7ms to be considered as vocalizations and those spaced by less than 50ms were grouped together. A final step of audio quality evaluation performed by an expert eliminated vocalizations that were contaminated by other vocalizations or noise on the microphone recording.

Acoustic features and modulation power spectrum (MPS)

28 predefined acoustic features (PAFs) were chosen to describe both the temporal and the spectral content of vocalizations. For each vocalization, PAFs were calculated from both the audiologger recording and from the ambient microphone recording. The signal captured by the piezo sensor of the audiologger was particularly suitable to measure pitch or amplitude related acoustic features because it is consistently placed very close to the larynx of the animal, the vocal source. The PAFs calculated from the audiologger signal were: duration, pitch saliency (a measure of how tonal vs noisy a sound is), fundamental frequency, coefficient of variation (CV) of the fundamental frequency, the five first momentum of the sound temporal envelope taken as a distribution (mean, standard deviation, kurtosis, skewness and entropy), the amplitude periodicity frequency (frequency peak in the amplitude power spectrum), the amplitude periodicity power index (ratio of the power at the amplitude periodicity frequency with the signal RMS), the five first momentum of the sound power spectrum taken as a distribution (mean, standard deviation, kurtosis, skewness and entropy) and the first, second and third spectral quartiles. The PAFs calculated from the microphone signal were: the five first momentum of the sound spectrum taken as a distribution (mean, standard deviation, kurtosis, skewness and entropy) and the first, second and third spectral quartiles. All these parameters except the two amplitude periodicity measurements were calculated using *soundsig* from the BioSound Python

441 library (https://github.com/theunissenlab/soundsig). The two amplitude periodicity
442 measurements were calculated using custom Matlab code.

443 The modulation power spectrum (MPS) of each vocalization was calculated from the
444 microphone signal using *soundsig* from the BioSound Python library
445 (https://github.com/theunissenlab/soundsig). The MPS is the 2D fast Fourier transform of the
446 sound spectrogram. To ensure that each vocalization MPS could be calculated at the same time
447 resolution, we ensured that each vocalization was isolated within a 100 ms window. After
448 bandpassing the microphone signal between 0.3 kHz and 50 kHz, the spectrogram was
449 calculated at a 1 kHz sampling rate using a frequency spacing of 100Hz. The spectrogram was
450 then z-scored and the MPS calculated using a window bin of 100ms. The MPS range was
451 focused on values with the most energy: the range of spectro-temporal modulations was
452 restricted from 0 to 3 cycles/kHz for the frequency modulations and from -150 Hz to 150 Hz for
453 the temporal modulations (see example MPS in Supplementary Figure 9). To account for the
454 various dynamic range across the spectro-temporal modulations, the MPS of each vocalization
455 was normalized by dividing with the average MPS over all vocalizations in the dataset to obtain
456 the Normalized Modulation Power Spectrum (NMPS, Supplementary Figure 9).

457 *UMAP projection and acoustic clustering*

458 The NMPS of the 28,091 vocalizations were reduced to fewer dimensions by applying the
459 algorithm UMAP (Uniform manifold approximation and projection) setting the number of
460 neighbors to 10, the minimum distance to 0.051 and the target weight to 0.55 (Matlab code
461 provided by the Herzenberg Lab at Stanford University, CA, USA). The NMPS as represented
462 by the UMAP values were then used as input to a hierarchical agglomerative clustering (HAC)
463 algorithm (*linkage* function of Matlab using the Ward method and the Euclidean metric) to divide
464 the acoustic space covered by the vocalizations into groups. The optimal number of dimensions
465 for the UMAP, the optimal tree cut and optimal number of acoustic groups were found by

466 measuring the average and median silhouette value over UMAPs of various dimensions and
467 various levels of tree cutting in the HAC (Supplementary Figure 10). Four acoustic groups in a
468 3D UMAP were found to be optimal numbers.
469
470 *Vocal dispersion*
471 Vocal dispersion is calculated for each individual bat as the generalized variance of its
472 vocalizations using the first 100 dimensions of the PCA on the NMPS of vocalizations from the
473 whole dataset. The first 100 dimensions explained 90% of the variance. The result depicted on
474 Supplementary Figure 5A, showing a larger vocal dispersion for males as compared to females,
475 was robust across all the number of dimensions tested (from 10 to 100 with a step of 10 and
476 from 100 to 300 with a step of 20).
477
478 *Acoustic dissimilarity (distance to opposite sex and distance to opposite hearing ability)*
479 The dissimilarity was calculated for each vocalization emitted by bat *i* as the distance to the
480 vocalizations from the nearest target bats *j* ($j \neq i$) in the space of the principal component
481 analysis (PCA) calculated on NMPS of vocalizations (Figure 2). Dimensions of the PCA had to
482 explain at least 1% of the variance to be included in this measure to ensure that the measure
483 would not be noise sensitive. As such the first 5 dimensions were retained. To represent how
484 males and females differentially occupy the acoustic space (Figure 2A), the target bats were the
485 opposite sex bats. To represent which vocalizations were affected by deafening (Figure 2B and
486 2D), the target bats were the opposite treatment but same sex bats. For each vocalization, the
487 acoustic dissimilarity is the distance to the closest of the target bats beyond what would be
488 expected given the local density of bats vocalizations. The distance from one particular
489 vocalization of bat *i* to the vocalizations of a *j* target bat was calculated as the average
490 Euclidean distance to the nearest 10 vocalizations of target bat *j*. The local density of bat

491 vocalizations (null distance) was the distance to any bat for the calculation of the sex
492 dissimilarity, and the distance to any same-sex bat for the calculation of hearing/deaf
493 dissimilarity. For a vocalization emitted by a male, the sex dissimilarity is the difference between
494 the average Euclidean distance to the 10 nearest vocalizations from the closest female bat, with
495 the average Euclidean distance to the 10 nearest vocalizations from any closest bat. For a
496 vocalization emitted by a hearing male, the hearing/deaf dissimilarity is the difference between
497 the average Euclidean distance to the 10 nearest vocalizations from the closest deaf male bat,
498 with the average Euclidean distance to the 10 nearest vocalizations from the closest male bat
499 (hearing or deaf). The proportion of the repertoire that was affected by deafening was estimated
500 as the proportion of vocalizations that displayed a hearing/deaf dissimilarity value larger than
501 the null distance to any same sex bats (Supplementary Figure 7).
502
503 *Regularized Permutation Discriminant Function Analysis*
504 The performance of classification of NMPS of vocalizations according to the hearing ability of
505 the emitter bat (Deaf and Hearing) was determined using a regularized permutation discriminant
506 function analysis (RPDFA). For each comparison (set of data), this procedure consisted in a
507 regularization achieved by a principal component analysis (PCA) on the NMPS, followed by a
508 Linear Fisher Discriminant Function Analysis (DFA). The performance of the DFA was
509 estimated in 10-fold cross-validation fixing the prior probability at 0.5. To find out the optimal
510 number of PCA dimensions to consider in the DFA, the performance of the classifier was
511 measured across a range of numbers of dimensions up to the number of dimensions that
512 explained 90% of the variance in the PCA (step of 10 up to 100 dimensions; step of 20 between
513 100 and 500 dimensions; step of 100 dimensions above). The optimal number of dimensions of
514 the PCA to consider in the DFA was found as the first value where the increase in classification
515 performance weighted by the increase of percentage of variance explained by the change of
516 number of dimensions is smaller than half the average standard deviation of the performance

517 across all values of dimensions tested (see Supplementary Figures 4, 6, 16 and 17). The
518 performance of the classifier at this optimized number of PCA dimensions was retained and
519 reported in the article. The chance level for the classification performance using the optimized
520 number of PCA dimensions was determined by running a permutation procedure where the
521 treatment of individuals was randomized across bats. Depending on the number of subjects in
522 the dataset there were 126 (ten male and female bats), 9 (six female bats) or 2 (four male bats)
523 different possible permutations. To determine if the odds of correct classification was higher in
524 the non-permuted treatment case than in the permutation cases, we perform a right tailed
525 Fisher's exact test for each permutation.
526 For display and interpretation purposes, the DFA at the optimal number of PCA dimensions was
527 recalculated without cross-validation to obtain the discriminant function as shown in Figure 3
528 and Supplementary Figure 11.
529

530 ## Data availability

531 The database of calls will be made available on FigShare upon publication.
532

533 ## Code availability

534 All the code used for call detection, curation and analyses is available under Github.
535

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537

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Author contributions

Conceptualization: MMY, SEM, JEE

Methodology: MMY, SEM, JEE, YR, LAB, DLS

Resources: MMY, YR

Data Curation: JEE

Investigation: SEM, JEE, DG, LAB

Software: JEE, TN

Formal acoustical analysis: JEE

Formal histological analysis: LAB, DLS, YR

Funding acquisition: MMY

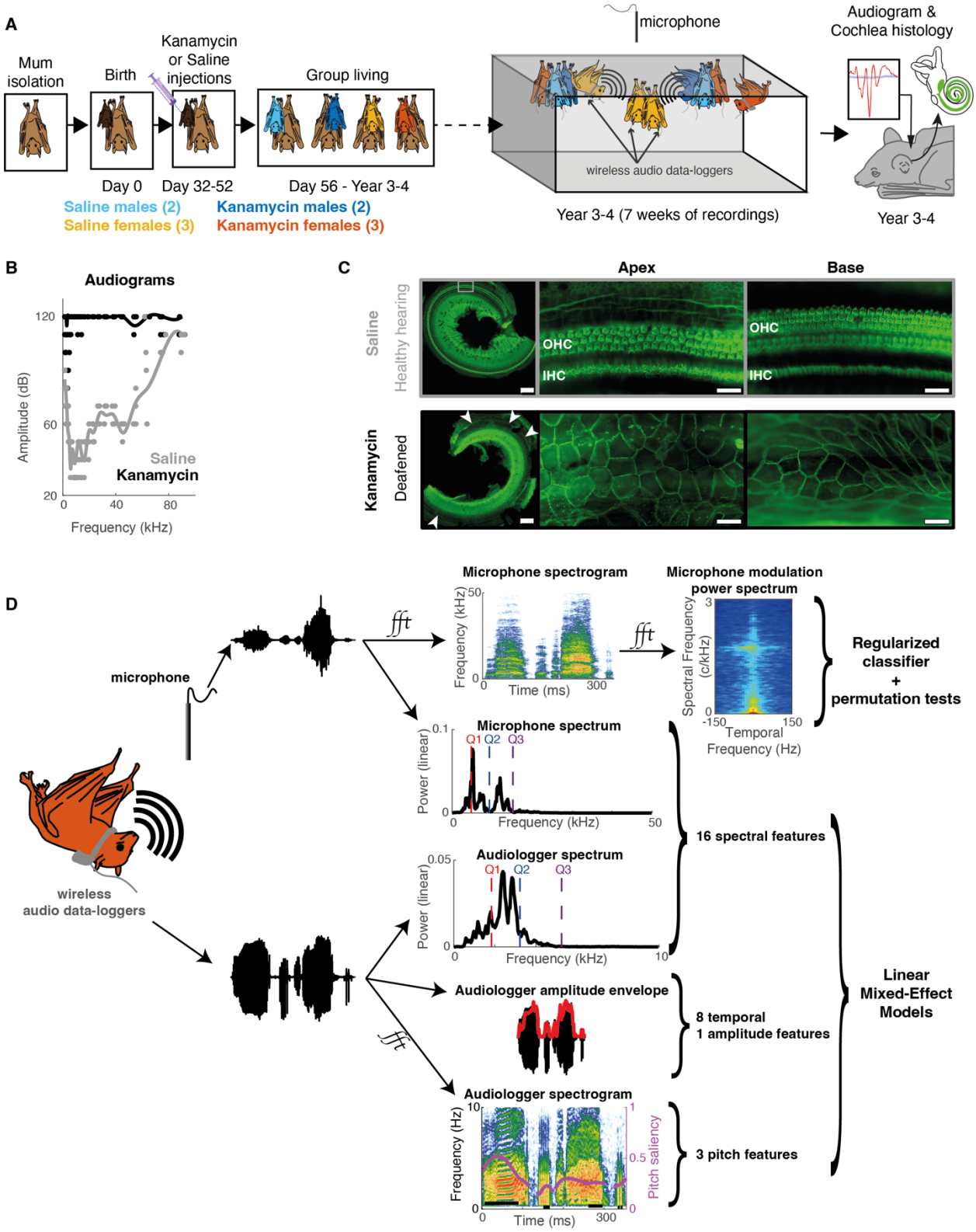
Supervision: JEE, MMY

Writing – original draft: JEE

Writing – review & editing: JEE, MMY, LAB, TN, SEM, DG, YR, DLS

Competing interests

The authors have no competing interests to declare.


A
Mum isolation
Birth
Kanamycin or Saline injections
Group living
Day 0
Day 32-52
Day 56 - Year 3-4
Saline males (2)
Saline females (3)
Kanamycin males (2)
Kanamycin females (3)
microphone
wireless audio data-loggers
Year 3-4 (7 weeks of recordings)
Audiogram & Cochlea histology
Year 3-4
B
Audiograms
Amplitude (dB)
Frequency (kHz)
Saline
Kanamycin
C
Apex
Base
Saline
Healthy hearing
Kanamycin
Deafened
OHC
IHC
D
microphone
wireless audio data-loggers
fft
Microphone spectrogram
Frequency (kHz)
Time (ms)
Microphone modulation power spectrum
Spectral Frequency (c/kHz)
Temporal Frequency (Hz)
Regularized classifier + permutation tests
Microphone spectrum
Power (linear)
Frequency (kHz)
Q1 Q2 Q3
Audiologger spectrum
16 spectral features
Audiologger amplitude envelope
8 temporal
1 amplitude features
Linear Mixed-Effect Models
Audiologger spectrogram
Frequency (Hz)
Pitch saliency
3 pitch features

644 Figure 1 Experimental design testing the necessity of hearing from birth for the normal
645 development of the vocal repertoire in adult Egyptian fruit-bats
646 A. Pregnant females gave birth and cared for their young in complete isolation from the rest of
647 the colony for two months. Their ten pups were randomly assigned to a treatment by kanamycin
648 or saline (control). Mothers and pups were then reunited with the rest of the colony for 3-4
649 years. Well after reaching adulthood, the ten manipulated bats were housed together and their
650 adult vocal behavior was recorded for seven weeks. Finally, their audiograms were measured
651 and their cochlea inspected for histological damage.
652 B. Audiograms obtained from measuring the Auditory Brainstem Response of all bats (n=10) to
653 tone pips covering the species auditory range. Dots depict individual values, while lines show
654 the average fitted with a spline. Note that responses of the auditory nerve were absent (values
655 ceiled at 120dB) or only detectable for very loud sounds (>90dB) in kanamycin treated bats,
656 while the expected audiogram was obtained for saline treated bats.
657 C. Representative examples of the hearing sensory epithelium (organ of Corti) at two levels of
658 the cochlea (apical turn, first and second columns; basal turn, third column) in two bats stained
659 with phalloidin (first row, female; second row, male). Scale bars: 200μm in first column, 25μm in
660 other columns; grey square: area shown in second column. Note the intact epithelium (OHC,
661 outer hair cells; IHC, inner hair cells) both in the apical and basal cochlear areas of the saline
662 bat, as opposed to the non-sensory flat epithelium in the kanamycin bat. Hair cells are absent
663 throughout the organ of Corti (see white arrows) except for a few cells in kanamycin female bats
664 (Supplementary Figure 1).
665 D. Vocalizations are recorded both from an environment microphone and a piezo sensor placed
666 against the throat of the bat (wireless audiologger). Two approaches are used to find the effect
667 of deafening on bats' vocalizations. Upper panel: the data driven approach is using the
668 modulation power spectrum of vocalizations as input to a classifier that is trained and tested in

669 cross-validation to discriminate between hearing and deaf bats' calls. The permutation test gives
670 the correct chance level taking into account any acoustic differences due to bat identity.
671 The second approach test the effect of deafening by fitting a Linear Mixed-Effect model
672 controlling for the identity of the bats on each of 28 acoustic features of the vocalizations
673 (Supplementary Figure 3 and methods).

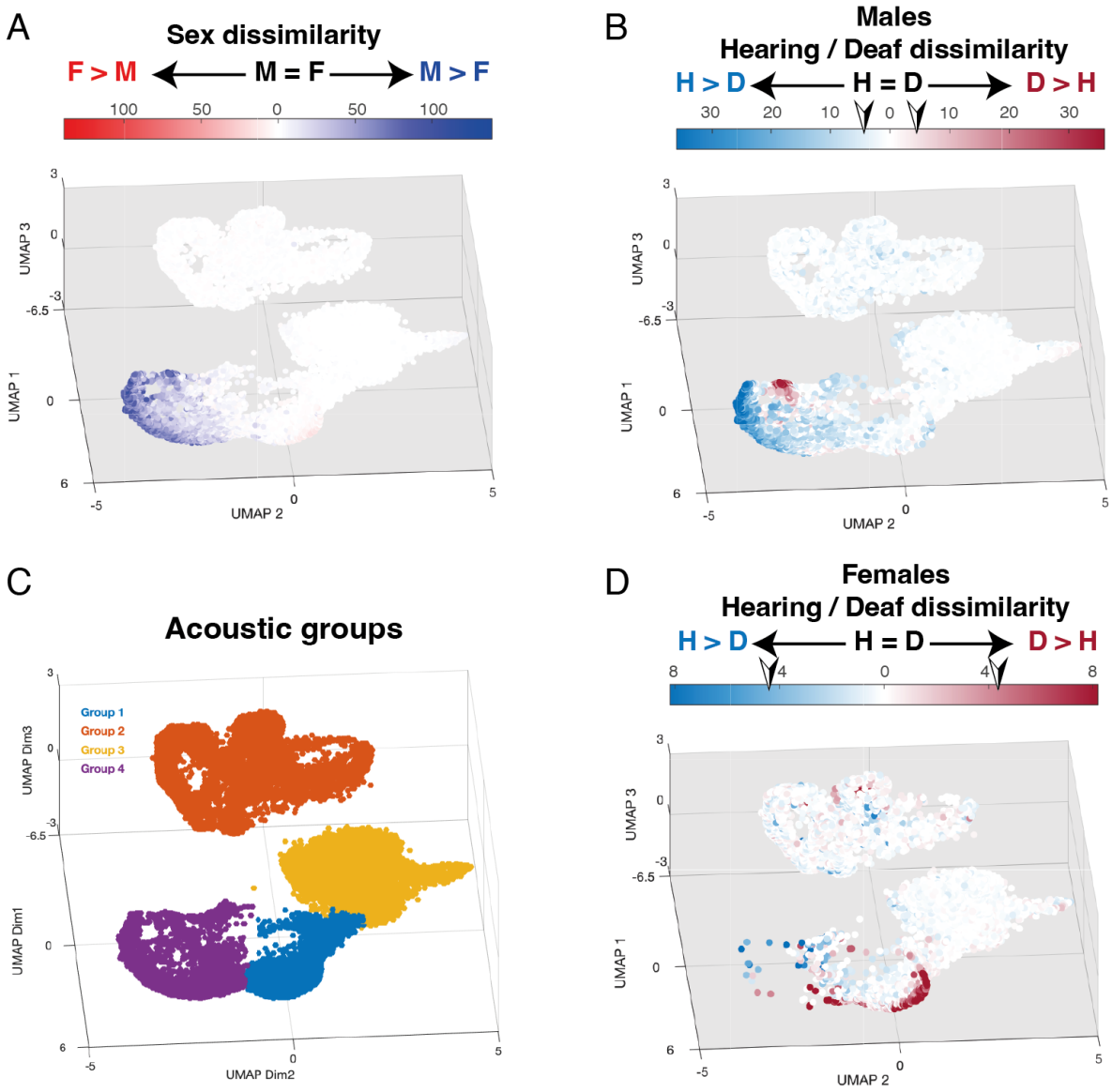


Figure 2 Effect of sex and deafness through the vocal space

Projections of vocalizations as represented by their MPS in a 3-dimension UMAP, the vocal space.

A. Dissimilarity of bat vocalizations to opposite sex vocalizations. The color of the points indicate the sex of the emitter (male, blue, and female, red) and the intensity of the color the linear PCA distance to the closest ten vocalizations produced by an opposite sex bat (see methods). Note that vocalizations with UMAP2<-2 are almost exclusively produced by males.

B and D Dissimilarity of male (B) or female (D) vocalizations to vocalizations from a same-sex

683 bat with opposite hearing ability. Male (B) or female (D) vocalizations are color coded according
684 to the hearing ability of the emitter (hearing, blue, and deaf, red). The intensity of the color
685 corresponds to the linear PCA distance to the closest ten vocalizations produced by a male (B)
686 or a female (D) of opposite hearing ability (methods). The arrowhead on the color scale
687 indicates the threshold over which vocalizations were considered idiosyncratic of the bat hearing
688 ability (dissimilarity value larger than expected on average to any same sex calls; see
689 Supplementary figure 8 and methods).
690 C. Parsing of the vocal space into four groups according to the results of a Hierarchical
691 Agglomerative Clustering algorithm.

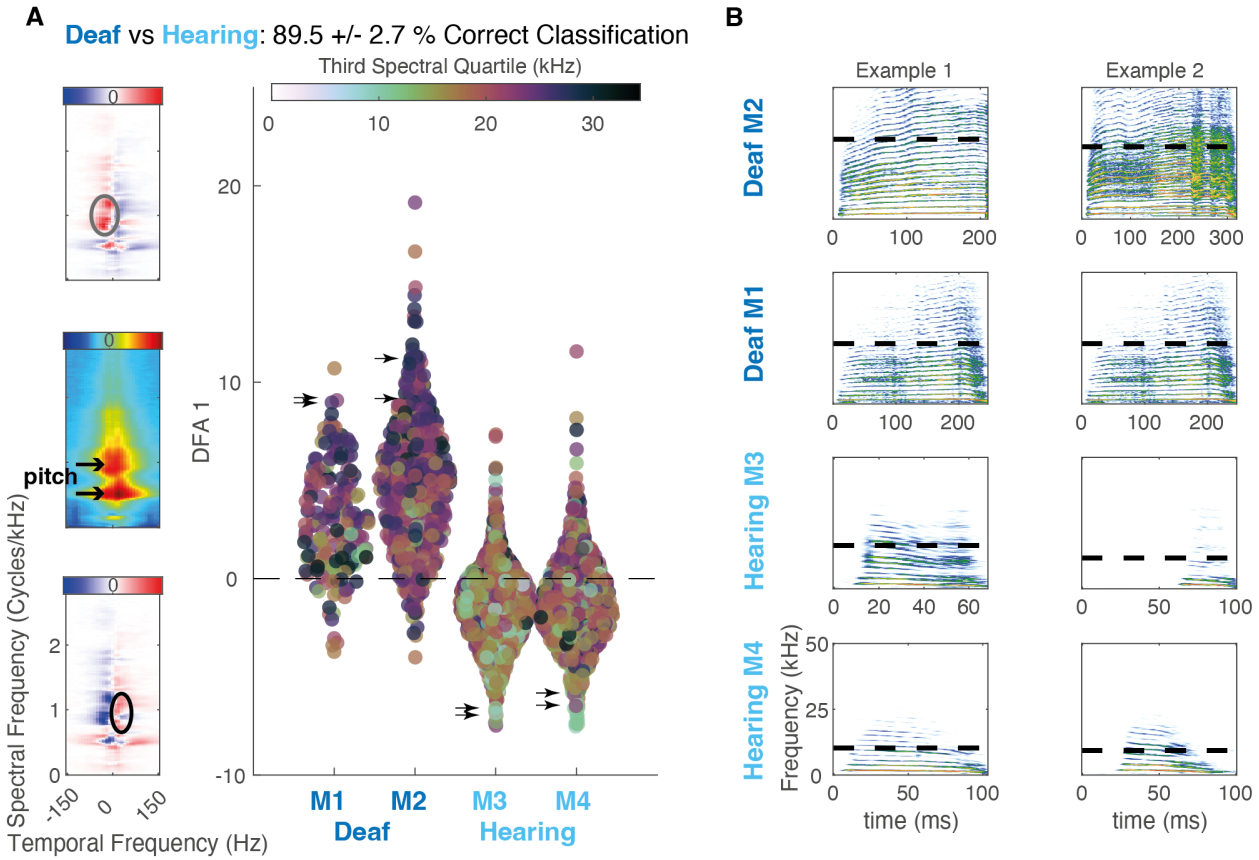


Figure 3 Effect of deafness on male calls in acoustic group 4

A. Coefficients of the acoustic Group 4 vocalizations of each male bat when projected on the first linear acoustic axis of the PCARDFA that best discriminates between deaf (M1 and M2) and hearing (M3 and M4) bats. Each circle in the scatter plot represents a vocalization. The circle color indicates the value of the Third Spectral Quartile of the vocalization. The three images on the left show the average MPS of male vocalizations in acoustic Group 4 (middle) and the spectro-temporal changes in the MPS (discriminant function) that best characterize vocalizations from deaf males (top) and vocalizations from hearing males (bottom). Note that while on average male vocalizations in acoustic Group 4 are characterized by pitch values of 1c/kHz or 0.5 c/kHz corresponding respectively to 1kHz and 2kHz (black arrows), the time

varying pitch modulations are different between deaf and hearing males: hearing males produce calls with more downsweeps (black circle) and deaf males with more upsweeps (gray circle). The black arrows in the scatter plot point to the example calls shown in B.

B. Spectrogram of two example calls from acoustic Group 4, per male bat. Note that hearing male vocalizations are short downsweep vocalizations with a low Third Spectral Quartile (black dotted lines) while the deaf male vocalizations are longer and more pitch modulated vocalizations (presence of upsweeps) with high Third Spectral Quartile (black dotted lines). The audio file of each of these examples can be found as supplementary materials (Group4_DeafMaleBatM2_1.wav, Group4_DeafMaleBatM2_2.wav, Group4_DeafMaleBatM1_1.wav, Group4_DeafMaleBatM1_2.wav, Group4_HearingMaleBatM3_1.wav, Group4_HearingMaleBatM3_2.wav, Group4_HearingMaleBatM4_1.wav, Group4_HearingMaleBatM4_2.wav)