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Vocalization Development in Geoffroy's bat, Myotis emarginatus (Chiroptera: Vespertilionidae)

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Postnatal development of vocalization has been studied in a small number of bats, not including the Geoffroy's bat, *Myotis emarginatus*. In the present study, we investigated vocalization development at a maternity roost of *M. emarginatus* in Kerend Cave in western Iran by sequential measurement of acoustic parameters in known-age neonates using mark-recapture sampling. Newborn pups of *M. emarginatus* produced both short and isolation calls. Duration of short calls of 1-day-old pups was on average 7.56 \pm 0.05 ms and decreased during postnatal development to reach an adult-like duration of 2.78 \pm 0.13 ms in the third week. *Myotis emarginatus* emitted various types of isolation calls, including classic, UP-tail-FM and CF-tail during postnatal growth. As bat pups grew, both short and isolation calls changed in their spectro-temporal structure. Discriminant function analyses showed that each bat pup has a vocal signature that facilitates mother-infant communication. The current study reveals that *M. emarginatus* pups are highly vocal at birth, but gradually elaborate their sounds with an increase in peak, start and end frequencies as well as with a decrease in call duration throughout the postnatal growth period.

Key words: Echolocation, Frequency-modulated, Syllable, Isolation call, Development.

BACKGROUND

Bats are among the most vocal and social mammals, with vocalization behaviour in both males and females, applied as a strong social interaction method (Fenton 2003; Riccucci 2011; Schuchmann et al. 2012). Bat pups are also highly vocal, producing varieties of vocalization that include isolation calls and echolocation call precursors. Pups produce isolation calls when they are separated from their mothers and seeking maternal care (Bradbury and Vehrencamp 2011; Knörnschild et al. 2013; Engler et al. 2017). Several studies have demonstrated that bat pups can distinguish between calls from their own mothers and those from other females (Eptesicus fuscus, Mayberry and Faure 2015; Glossophaga soricina, Engler et al. 2017; Saccopteryx bilineata, Fernandez and Knörnschild 2017). Likewise, adult bats can discriminate between vocalizations of pups and adults. When searching for pups, female bats produce pulses with high repetition rate known as directive calls (Balcombe and McCracken 1992). Directive call structure and context varies with echolocation calls by having lower frequencies (Bohn et al. 2008). For example, adult females of *Phyllostomus hastatus* are able to identify their pups' isolation calls (Bohn et al. 2007), implying that individual acoustic signature is critical in mother-young communication and recognition (Bohn et al. 2007; Knörnschild and von Helverson 2008).

At birth, vocalization signals of bat pups are characterized mostly by calls of low frequency, multiharmonic, with specific temporal pattern (Monroy et al. 2011; Wang et al. 2014) and the ability to encode information about the individual (Knörnschild et al. 2012; Engler et al. 2017; Fernandez and Knörnschild 2017). Zhang et al. (2005), Jin et al. (2012) and Engler

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et al. (2017) distinguished between isolation calls and echolocation call precursors mainly based on call duration and defined the shortest calls as possible precursors of echolocation calls and those calls with longest pulse duration as isolation calls. Isolation calls are more variable in their spectro-temporal features compared with echolocation call precursors (Sterbing 2002; Bohn et al. 2008; Monroy et al. 2011). Isolation calls are usually recognized and characterized by specific acoustic properties of individuals that are critical for conveying information to mothers, referred to as syllables (Monroy et al. 2011; Fernandez and Knörnschild 2017). As bat pups proceed with growth stages from infancy to adulthood, vocalization signals develop. In several bat species, pup isolation calls are precursors of both echolocation and communication calls (e.g., Carollia perspicillata, Sterbing 2002; Rhinolophus ferrumequinum, Andrews et al. 2011; E. fuscus, Monroy et al. 2011; Myotis macrodactylus, Wang et al. 2014). According to the majority of the studies on bat vocalization development, pups produce not only isolation calls but also echolocation call precursors (e.g., Tylonycteris pachypus and T. robustula, Zhang et al. 2005; Nyctalus noctula, Knörnschild et al. 2007; Hipposideros pomona, Jin et al. 2011; Vespertilio sinensis, Jin et al. 2012; G. soricina, Engler et al. 2017; Myotis capaccinii, Mehdizadeh et al. 2018).

In a comparison with other Myotis species, Myotis emarginatus (the Geoffroy's bat, Geoffroy 1806) is characterized by its relatively small body size and ears with a distinct notch, hence its common name is notch-eared bat (DeBlase 1980). Myotis emarginatus (type locality: France, Ardennes, Charlemont) is typically distributed in the north-west Africa and western Europe through Central and Mediterranean Europe to the Caucasus, southern Arabia, the eastern part of the Mediterranean, Iran, Afghanistan and West Turkestan (Benda et al. 2012). In the distribution range of the species, *M. emarginatus* is represented by two subspecies based on morphological studies: M. e. emarginatus and M. e. desertorum (Dobson 1875). The nominotypical species, M. e. emarginatus, is small to medium in size and occurs in the Maghreb, southwestern and central Europe and the Balkans to the Levant, and M. e. desertorum, large in size, occurs in Crimea, Transcaucasia, Iran, Afghanistan and West Turkestan (Benda et al. 2006). The species has been recorded from different parts of Iran (DeBlase 1980), including the Alborz Mts., mountainous parts of north-eastern Khorasan, the northern and southwestern Zagros Mts., the eastern Baluchistan and the Hormozgan Plain (DeBlase 1980; Benda et al. 2012). The IUCN classified the global conservation status of M. emarginatus as "Least Concern" (Piraccini 2016).

Myotis emarginatus produces frequency modulated (FM) echolocation calls, often with a very high start frequency, over 140 kHz (Dietz et al. 2007). The ranges of peak frequency have been reported between 43–87 kHz from the European populations (Papadatou et al. 2008). Call recordings of the species have not yet been reported in Iran; however, the range of peak frequency has been between 46–50 kHz in Oman (Benda et al. 2010). Postnatal growth in body mass and flight-related morphological features were simultaneously studied by mark-recapture method in *M. emarginatus* in Kerend Cave (Eghbali and Sharifi 2018).

The present study is the first attempt in examining the vocal repertoire of M. *emarginatus* and testing whether echolocation calls are developed parallel to isolation calls. Accordingly, we aimed to: (1) examine if there are age differences in acoustic features, (2) describe acoustic signals in the bat pups of M. *emarginatus* at different stages of vocalization development and (3) check whether each individual pup has its own individual-specific signature of isolation calls.

MATERIALS AND METHODS

Study Site

Our study focused on a nursery colony of *Myotis* emarginatus at Kerend Cave (34°15'N and 46°17'E) in western Iran. The cave is located on the eastern side of the Kerend town, Kermanshah Province in the mid-Zagros Range. The climate in the western edge of the Iranian Plateau is characterized by a distinct seasonal variation which includes a long freezing period in winter and a mild summer. For the period 1987–2014, the annual mean rainfall and temperature in the area were 462.23 ± 13.00 mm and 14.18 ± 0.92 °C, respectively (Eghbali and Sharifi 2019). The landscape around the cave is characterized by rough topography, very shallow soil, large extent of rock cliffs and sparse vegetation cover.

Recording and Analysis of Calls

The marking, recapturing and sound recording of young bats took place while adults were out foraging. We continued to recapture bat pups until their vocal repertoire became similar to those of adults. Sampling for vocalizations was taken every 4–7 days comprising a total number of seven samples for sound recording. In total, 51 pups were subjected to sound recording, of which 21 groups of mother and pups were included. Vocalizations were recorded from each pup on seven

occasions from 3 to 29 June 2016. In order to maintain bat body odour and avoid rejection of pups by their mothers, we used individual surgical gloves when handling the neonates. Upon capture, the pups were placed individually in white cloth bags (30×40 cm) and following sex determination, the forearm of new-born pups was tagged with a small numbered aluminium tag of 2.9 mm and 0.05 g (Porzana, Co. Ltd. Norfolk, UK). The 1-day-old pups were identified with an umbilical cord attached (Kunz and Robson 1995). Calls produced by *M. emarginatus* pups were recorded and analyzed outside the cave in a relatively silent environment. To test condition-related behaviour in bat pups, they were isolated and their behavioural response to the separation from the mother was examined.

We used a Pettersson D240X bat detector (Pettersson Elektronik AB, Uppsala, Sweden; a sampling frequency of 44.1 kHz with 16 bits/sample) with a digital audio recorder (Edirol, Poland) connected in time expanded (x10) mode. Auto trigger mode switched to the position of high level and Hf socket. The bat detector was placed 10–15 cm from bats. Call recordings were made when they were brought out from the cloth bag. Vocalization was recorded from pupmother pair at the time of separation and reunion. Bat pups were brought back to their roost after assessing motion, physical status and call recording. Multiple clear vocalizations were selected from each individual and processed using the software BatSound Pro ver. 3.31 (Pettersson Elektronik AB, Uppsala, Sweden).

Temporal and spectral analyses of the oscillogram and spectrogram were conducted. The spectrogram depicts frequency as a function of time and was generated using a 512-point fast Fourier transform, a Hanning window with 75% overlap (frequency resolution: 1206 Hz and time between FFTs: 0.29 ms). The calls were multi-harmonic and the maximum number of harmonics was four. We only used the first harmonic (fundamental harmonic) for measurements since it contained the most energy in the call (Vespertilio sinensis, Jin et al. 2012; Myotis capaccinii, Mehdizadeh et al. 2018). The tonal calls (every single syllable) were classified into two types-short and isolation callsbased on their auditory quality, visual inspection of spectrograms and duration of calls emitted at birth that were different from 7 to 40 ms. Therefore, the longest calls are considered isolation calls and the shortest calls were considered precursors of echolocation.

The types of isolation calls were also defined according to spectro-temporal features of the tail part of every syllable. The tail part of a syllable is a part that ends in CF, UP and sinusoid the tail. Isolation calls include single- and double-syllable calls. Single-syllable calls include classic, sinusoid, CF-tail and UP-tail-FM calls and double-syllable calls include FM and FM, CF-tail and FM and UP-tail-FM and FM. The classic isolation calls are FM syllables with slow downward sweeps and no tail. The sinusoid isolation calls are similar to classic isolation calls, which follow with a sinusoid-like tail. The UP-tail-FM isolation calls are FM syllables with very slow downward sweeps and a tail that is an FM syllable with upward sweeps. The CFtail isolation calls are the initial FM part with very short downward sweeps that follow with very long constant frequency component. The first and second syllables of double-syllable calls are similar to single-syllable calls. Four parameters were selected as attributes of vocalizations: CD [call duration in millisecond (ms) between time of the start and end of pulse in the oscillogram], PF [peak frequency in kilohertz (kHz) with greatest amplitude in the power spectrum], SF [start frequency (kHz) of the pulse in the spectrogram] and EF [end frequency (kHz) of the pulse in the spectrogram].

To examine whether the structure of isolation calls varied among individuals, a linear discriminant function analysis (DFA) was conducted on calls produced by five bats at 1-day-old and 15 bats at 4-day-old and analysed at least in 10 isolation calls (10-20 calls per pup, 73 and 173 calls in total for 5 and 15 pups, respectively). Analyses were carried out with commercial statistics software SPSS (v.16.; Chicago: SPSS Inc) and Excel 2013. The data were presented as mean \pm SE (standard error) unless otherwise stated. In this study, the terms "bout", "call" and "syllable" are used based on terminology applied by Gould (1975), Bohn et al. (2008) and Hechavarría et al. (2016). A syllable is defined as an acoustic unit of a vocalization surrounded by silence. The call is defined as the simplest emission of a vocalization that can be represented as multisyllabic (composed of different monosyllabic calls) or monosyllabic (one type of syllable or similar syllables). Bout is considered a group of calls. The development stages of vocalization were defined after Powers et al. (1991), Funakoshi et al. (2010) and Mehdizadeh et al. (2018): 1) Flopping stage (new-born), bats are unable to fly actively; 2) Fluttering stage, bats can form a colony and may be able to move short distances by fluttering; 3) Flapping stage, bats may be able to fly inside the cave by wing flapping; 4) Flying stage, bats are able to keep balance during flight inside the cave.

RESULTS

Echolocation and Directive Calls of Adult Females

Echolocation and directive calls produced by 12

adult female individuals of *Myotis emarginatus* were recorded and analysed. The echolocation calls mostly consisted of one harmonic that started with a short and steep FM call (Fig. 1). The values of start, end and peak frequencies recorded in the echolocation calls lasted 1.60 ± 0.16 ms and were 138.80 ± 1.79 kHz, $41.50 \pm$ 0.88 kHz and 92.20 ± 2.61 kHz, respectively. Directive calls of female bats consisted of several calls occurring in rapid succession, with each bout typically consisting of 4–16 calls (Fig. 1). The start (SF), end (EF) and peak (PF) frequencies of *M. emarginatus* directive calls lasted 1.58 ± 0.15 ms and were 51.33 ± 2.20 kHz, $16.92 \pm$ ± 0.72 kHz and 36.00 ± 0.90 kHz, respectively.

Flopping Stage

During the first 4-5 days, new-born M. emarginatus pups emitted a variety of vocalizations most of which are audible (isolation call ~85%), but some are ultrasonic short calls (~15%). The multiharmonic calls were mostly single syllable downward frequency-modulated (FM) sweeps in isolation calls and short calls. The shortest vocalizations were considered precursors of echolocation calls. In 1-day-old new-born bats, short calls were FM syllables with rapid downward sweeps (7.56 \pm 0.05 ms). These calls contained four harmonics and the fundamental harmonic swept from about 29.38–18.24 kHz with a peak energy frequency of approximately 25.37 kHz (Fig. 2, Table 1). The SF, EF and PF of 4-day-old pups lasted 4.28 ± 0.31 ms and were 41.59 \pm 1.89 kHz, 20.89 \pm 0.95 kHz and 36.69 \pm 0.68 kHz, respectively (Fig. 2, Table 1).

At birth, the pups in the presence and absence of

their mothers emitted long calls that lasted $\sim 14-36$ ms. These calls were considered isolation calls. The vocalizations were different in spectro-temporal features of the tail part of the call that followed downward frequency-modulated sweep of each syllable. The types of isolation calls for 1-day-old pups included the following: (I) classic isolation calls were FM syllables with slow downward sweeps (19.34 ms) and 3-4harmonics with no tail (Fig. 3), (II) UP-tail-FM isolation calls were FM syllables with very slow downward sweeps (34.45 ms) with tail that was FM syllable with upward sweeps (14.72 ms) and 3-4 harmonics (Fig. 2), (III) CF-tail isolation calls were the initial FM part with very short downward sweeps (18.00 ms) that followed with very long constant frequency component (35.20 ms), and (IV) two isolation calls which seem to be a preface for double-syllable isolation calls in the next stages of call development.

For 4-day-old pups, the types of isolation calls are described as follows: (I) classic isolation calls mostly become visible to sinusoid like tail; (II) UP-tail-FM isolation calls, the initial FM component (12.40 ms) which followed with FM upward sweep tail (9.60 ms); (III) CF-tail-isolation calls whose duration of initial and second component were 17.20 ms and 23.20 ms; and (IV) double-syllables that were rarely seen, the first and second syllables of which were 12.10 ms and 15.30 ms, respectively. At this point, most of the pups' vocalizations were classic isolation calls and in general, start, end and peak frequencies of isolation calls types (UP-tail-FM and CF-tail) were nearly identical (SF: 38.71 ± 3.88 kHz, PF: 29.42 ± 4.02 kHz, EF: 18.79 ± 3.38 kHz; Fig. 3, Table 2).



Fig. 1. Typical echolocation and directive calls of an adult Myotis emarginatus.



Fig. 2. Spectrogram of short calls produced by Myotis emarginatus pup from day 1 to 27 recorded in seven sampling occasions.



Fig. 3. Spectrogram of vocalization types of isolation calls in *Myotis emarginatus* pups, which include single-syllable (classic, sinusoid, CF-tail and UP-tail-FM) and double-syllable (FM and FM, CF-tail and FM and UP-tail-FM and FM) calls.

Table 1. Developmental changes of the short call in start frequency (SF), end frequency (EF), peak frequency (PF), call duration (CD) and number of harmonics (NH) in *Myotis emarginatus* pup with mean \pm SE. *n*, number of bat pups which were recorded

Vocal features	Age									
	1st week old		2nd weeks old		3rd weeks old		4th weeks old			
	1 day (n = 5)	4 days $(n = 3)$	8 days (<i>n</i> = 17)	12 days $(n = 6)$	16 days (<i>n</i> = 13)	20 days (<i>n</i> = 5)	27 days $(n = 4)$			
SF (kHz)	29.38 ± 2.07	41.59 ± 1.89	62.94 ± 2.12	95.82 ± 3.90	112.32 ± 3.32	132.80 ± 2.61	128.92 ± 3.87			
EF (kHz)	18.24 ± 2.20	20.89 ± 0.95	27.71 ± 0.83	39.54 ± 0.90	41.31 ± 0.93	43.05 ± 1.36	40.40 ± 1.08			
PF (kHz)	25.37 ± 2.00	36.69 ± 0.68	49.75 ± 1.75	77.40 ± 5.20	82.87 ± 1.72	88.73 ± 1.64	89.93 ± 3.06			
CD (ms)	7.56 ± 0.05	4.28 ± 0.31	3.42 ± 0.18	2.38 ± 0.47	2.78 ± 0.13	2.48 ± 0.17	2.23 ± 0.18			
NH	4	3–4	2–3	2	1–2	1	1			

Fluttering Stage

During the first two weeks after birth, pups were very vibrant, with some walking rapidly on the ceiling

and some showing fluttering activity (falling with wing flapping, but not able to perform horizontal flight). At this stage, most pups ($\sim 80\%$) emitted short calls as shown in figure 2. Short calls in 12-day-old pups

Table 2. Descriptive statistics for time and frequency parameters [start frequency (SF), end frequency (EF), peak frequency (PF) and call duration (CD)] of the isolation calls of single-syllable (classic) and double-syllable including FM and FM (the first syllable, [I] and the second syllable, [II]) and UP-tail-FM and FM (the first syllable, [I] and the second syllable, [II]) in *Myotis emarginatus* pup with mean \pm SE. *n*, number of bat pups which were recorded

Isolation calls		Age								
			lst we 1 day	eek old 4 days	2nd wo 8 days	eek old 12 days	3rd w 16 days	eek old 20 days	4th week old 27 days	
Classic		SF	32.19 ± 1.18	38.71 ± 1.00	66.25 ± 0.85	90.50 ± 1.32	111.40 ± 2.36	131.33 ± 2.40	135.00 ± 3.51	
			<i>n</i> = 5	<i>n</i> = 15	<i>n</i> = 4	<i>n</i> = 4	<i>n</i> = 5	<i>n</i> = 3	<i>n</i> = 3	
		EF	17.12 ± 0.83	18.79 ± 0.87	26.75 ± 0.85	32.00 ± 2.68	37.40 ± 2.16	36.33 ± 1.20	41.33 ± 1.20	
			<i>n</i> = 5	<i>n</i> = 15	<i>n</i> = 4	<i>n</i> = 4	<i>n</i> = 5	<i>n</i> = 3	<i>n</i> = 3	
		PF	27.58 ± 1.27	29.42 ± 1.04	35.66 ± 0.70	42.65 ± 0.71	58.62 ± 1.77	89.00 ± 1.53	87.67 ± 4.05	
			n = 5	<i>n</i> = 15	<i>n</i> = 4	<i>n</i> = 4	<i>n</i> = 5	<i>n</i> = 3	<i>n</i> = 3	
		CD	19.34 ± 2.67	19.03 ± 0.85	12.10 ± 1.05	10.56 ± 1.50	4.08 ± 0.25	3.37 ± 0.22	3.40 ± 0.20	
			<i>n</i> = 5	<i>n</i> = 15	<i>n</i> = 4	<i>n</i> = 4	<i>n</i> = 5	<i>n</i> = 3	<i>n</i> = 3	
FM and FM	Ι	SF	-	-	67.50 ± 0.35	89.20 ± 1.47	74.33 ± 1.86	103.00 ± 3.00	131.00 ± 5.69	
					<i>n</i> = 2	<i>n</i> = 4	<i>n</i> = 3	<i>n</i> = 2	<i>n</i> = 3	
	II		-	-	62.50 ± 1.00	96.75 ± 2.10	99.00 ± 6.81	105.00 ± 0.00	134.33 ± 3.48	
					<i>n</i> = 2	<i>n</i> = 4	<i>n</i> = 3	<i>n</i> = 2	<i>n</i> = 3	
	Ι	EF	-	-	29.50 ± 0.50	27.00 ± 0.40	32.67 ± 0.88	39.50 ± 2.50	39.67 ± 1.20	
					<i>n</i> = 2	<i>n</i> = 4	<i>n</i> = 3	n = 2	<i>n</i> = 3	
	II		-	-	30.50 ± 2.50	36.00 ± 0.71	31.67 ± 2.73	38.50 ± 1.50	40.00 ± 1.53	
					<i>n</i> = 2	<i>n</i> = 4	<i>n</i> = 3	n = 2	<i>n</i> = 3	
	Ι	PF	-	-	36.70 ± 0.80	50.55 ± 1.82	51.23 ± 0.46	86.05 ± 0.95	86.00 ± 4.04	
					n = 2	<i>n</i> = 4	<i>n</i> = 3	n = 2	<i>n</i> = 3	
	Π		-	-	46.00 ± 3.00	49.50 ± 1.80	51.93 ± 0.33	88.00 ± 2.00	93.33 ± 3.28	
					n = 2	<i>n</i> = 4	<i>n</i> = 3	n = 2	<i>n</i> = 3	
	Ι	CD	-	-	14.55 ± 0.75	11.75 ± 0.84	6.33 ± 0.35	4.15 ± 0.05	3.90 ± 0.15	
					n = 2	<i>n</i> = 4	<i>n</i> = 3	n = 2	n = 3	
	II		-	-	5.25 ± 0.25	5.62 ± 0.33	4.20 ± 0.11	4.30 ± 0.30	3.20 ± 0.21	
					<i>n</i> = 2	<i>n</i> = 4	<i>n</i> = 3	<i>n</i> = 2	<i>n</i> = 3	
UP-tail-FM and FM	Ι	SF	-	-	67.33 ± 1.26	83.80 ± 0.10	89.00 ± 1.38	86.00 ± 2.52	88.50 ± 0.50	
					<i>n</i> = 6	<i>n</i> = 10	<i>n</i> = 5	<i>n</i> = 3	<i>n</i> = 2	
	II		-	-	62.50 ± 1.65	91.70 ± 0.60	108.00 ± 4.67	113.67 ± 3.28	116.00 ± 4.00	
					<i>n</i> = 6	<i>n</i> = 10	<i>n</i> = 5	<i>n</i> = 3	n = 2	
	Ι	EF	-	-	30.17 ± 1.96	38.70 ± 1.30	58.60 ± 4.92	50.33 ± 1.85	53.00 ± 1.00	
					<i>n</i> = 6	<i>n</i> = 10	<i>n</i> = 5	<i>n</i> = 3	n = 2	
	II		-	-	27.33 ± 1.78	30.00 ± 1.50	35.60 ± 1.60	38.33 ± 033	42.50 ± 0.50	
					n = 6	<i>n</i> = 10	<i>n</i> = 5	<i>n</i> = 3	n = 2	
	Ι	PF	-	-	34.80 ± 0.77	46.78 ± 1.77	48.76 ± 1.05	51.43 ± 3.61	71.00 ± 3.00	
					n = 6	n = 10	<i>n</i> = 5	<i>n</i> = 3	n = 2	
	II		-	-	42.27 ± 1.77	48.78 ± 1.37	53.26 ± 2.08	56.00 ± 2.00	80.00 ± 2.00	
					n = 6	n = 10	<i>n</i> = 5	<i>n</i> = 3	n = 2	
	Ι	CD	-	-	23.70 ± 1.25	19.92 ± 0.64	24.24 ± 1.59	17.33 ± 0.54	9.50 ± 2.00	
					n = 6	n = 10	<i>n</i> = 5	<i>n</i> = 3	n = 2	
	II		-	-	5.42 ± 0.40	6.08 ± 0.17	4.84 ± 0.72	6.23 ± 0.37	3.95 ± 0.05	
					<i>n</i> = 6	<i>n</i> = 10	<i>n</i> = 5	<i>n</i> = 3	n = 2	

had two harmonics. The SF and EF were on average 95.82 ± 3.90 kHz and 39.54 ± 0.90 kHz, respectively. Their call duration was on average 2.38 ± 0.47 ms (Fig. 2). Isolation calls were often emitted with changes in environmental conditions (e.g., separating from mother, encountering with mother following separation). When pups evoked, they would begin to emit several different types of isolation call (Fig. 3). Overall, most of the vocalizations were double-syllable calls and some single-syllable (Fig. 3). Single-syllable calls were mostly classic isolation calls. However, other forms of single-syllable calls (CF-tail and UP-tail-FM) were rarely emitted and values related to their parameters were similar to CF-tail and UP-tail-FM syllables of double-syllable phrases (Table 2). The classic isolation calls in this bat contained 2 to 3 harmonics. The average SF and EF of classic isolation calls were 90.5 \pm 1.32 kHz and 32.0 \pm 2.68 kHz, respectively, and call duration was 10.56 ± 1.50 ms. Double-syllable calls included FM and FM, CF-tail and FM and UP-tail-FM and FM. The call duration was long in the first syllable and average call duration in the UP-tail-FM component was 19.92 ± 0.64 ms. From this stage, the second syllable-frequency was mostly higher than that of the first syllable. For example, in FM and FM calls, start frequency of the first and second syllable in the 12-dayold individuals were $~89.20 \pm 1.47$ kHz and 96.75 \pm 2.10 kHz, respectively. Occasionally, a double-syllable call in the CF-tail was emitted, and the call duration of its tail part in a number of individuals was ~10 ms. The variations of peak frequency, start and end frequency were nearly similar to the short calls (Tables 1, 2).

Flapping Stage

At this stage, 16-day-old pups were able to perform a flapping flight inside the cave and their short calls were mostly a first harmonic and the call parameters were close to those of adults (SF: 112.32 \pm 3.32 kHz, PF: 82.87 ± 1.72 kHz, EF: 41.31 ± 0.93 kHz; Table 1). In stressful situations, pups also emitted a variety of isolation calls, including single- and doublesyllable of which most vocalizations were doublesyllable phrases. Some of the double-syllable phrases were FM and FM calls, but most were UP-tail-FM and FM calls. The average SF, EF and PF of classic isolation calls were 111.40 ± 2.36 kHz, 37.40 ± 2.16 kHz and 58.62 ± 1.77 kHz, respectively (Fig. 3). The frequency of FM and FM calls were 74.33 ± 1.86 kHz to 32.67 \pm 0.88 kHz and 99.0 \pm 6.81 kHz to 31.67 \pm 2.73 kHz, respectively. The values of average start to end frequencies in UP-tail-FM and FM calls were 89.00 \pm 1.38 kHz to 58.60 ± 4.92 kHz and 108.00 ± 4.67 kHz to 35.6 ± 1.60 kHz, respectively (Table 2).

Flying Stage

In this stage, 20-day-old pups emitted short calls that were similar to the echolocation calls of adults, with little change in frequency and duration (SF: 138.80 \pm 2.61 kHz, PF: 92.20 ± 1.64 kHz, EF: 41.50 ± 1.36 kHz; Fig. 2, Table 1). When the mother was introduced to her pup or the pups were evoked, they emitted calls with single, double and sometimes triple syllables. During this stage in 27-day-old pups, the tail component of UP-tail-FM syllables gradually disappeared and call duration decreased to 9.50 ± 2.00 ms. By week 3, the frequency of isolation calls syllables reached average adult values (SF: 135.00 \pm 3.51 kHz, PF: 87.67 \pm 4.05 kHz, EF: 41.33 ± 1.20 kHz; Fig. 2, Table 1) and the classic isolation calls emitted more than doublesyllabic calls (Table 2). In general, as pups matured, the call duration of isolation calls decreased considerably (Table 2).

Variation in Isolation Call Structure

Myotis emarginatus pups could be distinguished based on acoustic parameters of their isolation calls. A linear discriminant function analysis (DFA) was performed for 73 and 143 isolation calls in five (1-dayold) and 15 (4-day-old) pups, respectively. The call structure of individual pups consisted of a moderately short interval on the discriminant function, but considerable variations were found among individuals. The multiple discriminant analysis showed that 1-dayold pups could be distinguished when the call structure variables were linearly combined (Eigenvalue: DF1 = 7.134 and DF2 = 4.865, Wilks' λ : DF1 = 0.012 and DF2 = 0.095, all P < 0.0001). Discriminant function 1 and 2 explained over 91% of the variability in the variables measured (%Variation: DF1 = 55.80 and DF2 = 38.00). Call duration and end frequency gave the highest crosscorrelations with the discriminant function 1 and 2, respectively. By 4 days old, the multiple discriminant analysis also showed that pups could be distinguished based on their call structure (Eigenvalue: DF1 = 6.352and DF2 = 3.225, Wilks' λ : DF1 = 0.011 and DF1 = 0.082, all P < 0.0001). Again, the first two discriminant axes explained over 86.2% of the total variability in the variables measured (%Variation: DF1 = 57.20 and DF2= 29.00). End frequency and peak frequency gave the highest cross-correlations with discriminant functions 1 and 2, respectively.

DISCUSSION

This study is an attempt to show how Geoffroy's

bat pups emit acoustic signals from the early development until they start the first foraging flights. Bat pups emitted different types of multi-harmonic vocalizations that are longer in duration and lower in frequency than echolocation calls of adult bats. These can be confidently classified into precursors of echolocation signals (short calls) and communication signals (isolation calls). The results of our study are in agreement with those conducted on bat pups such as Pipistrellus pipistrellus (Jones et al. 1991), Noctilio albiventris (Brown et al. 1983), Pteronotus parnellii (Vater et al. 2003), Nyctalus noctula (Knörnschild et al. 2007), Hipposideros pomona (Jin et al. 2011), Artibeus jamaicensis (Carter et al. 2014), Glossophaga soricina (Engler et al. 2017) and Myotis capaccini (Mehdizadeh et al. 2018). Generally, these researchers have demonstrated that echolocation signals develop in parallel with communication signals. Accordingly, they argued that the organization of the neuronal circuits of echolocation and communication calls are controlled differentially in brain regions that are used for phonation (Fenzl and Schuller 2005; Metzner and Schuller 2010).

Based on the previous studies, we expected that the dominant calls during the first 4-5 days of birth would be isolation signals when bat pups needed the most maternal care and attention. Moreover, the structural properties of M. emarginatus isolation calls (*i.e.*, the spectro-temporal features of the call tail) were consistent with some previous reports on vocal development in bats (Monroy et al. 2011; Fernandez and Knörnschild 2017). Our data suggest that, starting in the second week of birth, the production of isolation calls decreased and the production of short calls increased. The ontogenetic change in acoustic signals is most probably the result of the maturation of vocal tracts such as larynx, vocal cords, trachea and muscles (Knörnschild et al. 2012). The short calls from the flopping stage to the flapping one gradually resembled adult echolocation signals and average peak frequency increased from 25.37 kHz to 82.87 kHz. By the end of the second week, short calls were similar spectrally and temporally to the echolocation calls emitted by adult M. emarginatus during foraging and navigation (Papadatou et al. 2008).

Furthermore, our results reflected that the echolocation calls of Geoffroy's bat pups developed at a more rapid rate than the communication calls (Figs. 2, 3). Using echolocation, most bats species navigate, locate and, capture their prey (Schnitzler and Kalko 2001) and since short and ultrasonic calls are precursors of echolocation calls, it can be concluded that precursors of echolocation calls of bat pups play roles in orientation and navigation, which will become more important for the survival of bats. In general, as bat pups grew,

both short and isolation calls changed in their spectrotemporal structure, which is consistent with the agerelated changes hypothesis (Jones et al. 1991; Moss et al. 1997; Zang et al. 2005; Jin et al. 2012; Wang et al. 2014; Mehdizadeh et al. 2018).

The development of isolation calls in bats is necessary for mother-infant and other social interactions (Knörnschild and Von Helversen 2008; Monroy et al. 2011; Knörnschild et al. 2013; Jin et al. 2015). According to the findings in the current study, the different types of isolation calls were emitted at developmental stages of M. emarginatus; these including types of single- and double-syllable isolation calls (Fig. 3). However, different types of isolation calls were emitted as the preface to complex communication calls (Monroy et al. 2011; Jin et al. 2015; Fernandez and Knörnschild 2017). Gould (1975) suggested that the particular calls are emitted at a level of excitation that is affected by various factors such as the age of a bat, its experience and stress levels. Fernandez and Knörnschild (2017) showed that different types of isolation call are emitted at different temporal stages. However, as M. emarginatus pup developed, call duration of the classic isolation calls decreased and call duration of isolation calls consisted of FM sweeps with tails also decreased slowly.

It is not possible to test playback experiments to clarify an individual-specific signature, but analysis of isolation call discrimination showed that classic isolation calls of 1-day-old and 4-day-old pups were individually specific and exhibited distinct spectrotemporal features. Isolation calls of bat pups carry individual-specific differences and can encode enough information to facilitate mother-offspring recognition. Such sound facilitation has been shown in several bat species (Phyllostomus hastatus, Bohn et al. 2007; Saccopteryx bilineata, Knörnschild and Von Helversen 2008; M. lucifugus, Melendez and Feng 2010; Carollia perspicillata, Knörnschild et al. 2013; G. soricina, Engler et al. 2017; S. bilineata, Fernandez and Knörnschild 2017). Since the new-born bats are altricial (Kurta and Kunz 1987) and their survival depends critically on maternal care, the individual signature can potentially be used as a survival factor to attract the mother's attention (Bohn et al. 2007; Knörnschild and Von Helversen 2008; Knörnschild et al. 2012; Engler et al. 2017).

Several species belonging to the family Vespertilionidae have evolved the ability to emit a wide variety of isolation sounds at birth. Some of these species—including *M. lusifugus* (Buchler 1980), *Plecotus auritus* (De Fanis and Jones 1995), *Vespertilio sinensis* (Jin et al. 2012) and *M. macrodactylus* (Wang et al. 2014)—emit multi-harmonic isolation calls with

the FM structure with slow downward sweeps during vocalization development, while other species, such as P. pipistrellus (Jones et al. 1991), Eptesicus fuscus (Monroy et al. 2011; Mayberry and Faure 2015) and M. emarginatus in the present study, emit multiharmonics isolation call with different spectral and temporal characteristics in the tail part of the sound. In addition, C. perspicillata (Phyllostomidae, Sterbing 2002), Glossophaga soricina (Emballonuridae, Engler et al. 2017) and Saccopteryx bilineata (Emballonuridae, Fernandez and Knörnschild 2017) emit different types of isolation calls during vocalization development. However, Miniopterus fuliginosus (Miniopteridae, Funakoshi et al. 2013) emits only a single type sound, which is a precursor to echolocation and isolation calls. These differences can be generally due to the structural development of the larynx (Fattu and Suthers 1981; Carter and Adams 2016).

CONCLUSIONS

This study evaluated the development of vocalization behaviour in a free ranging population of the Geoffroy's bat, M. emarginatus, during 27 days of postnatal period from birth until the first foraging flights. In our study, we surveyed how Geoffroy's bat pups emitted acoustic signals from early development until they started foraging outside the cave. Bat pups emitted different types of multi-harmonic vocalizations that were longer in duration and lower in frequency than echolocation calls of adult bats. These calls can be categorized into precursors of echolocation signals (short calls) and communication signals (isolation calls). This study also highlighted several types of isolation signals that were expressed temporally and provided different information layers decoded in single and composite syllables. Finally, future studies could use playback experiments to examine the exact role of isolation calls in determining parental care, individual signature and mother-offspring interaction.

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