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Is species identity, sex, age or individual quality conveyed by echolocation call frequency in European horseshoe bats?

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Horseshoe bats (*Rhinolophus*) use echolocation calls with a prominent part whose frequency is constant over time (CF) and matches the 'acoustic fovea' of the bats' hearing system. The present study on European *Rhinolophus* species investigates whether this CF component contains reliable information on species, sex, age class or quality (size and body condition) of the caller and could therefore have a communicative value. The resting frequencies (RF) were measured from stationary, handheld bats for the species *Rhinolophus blasii*, *R. euryale*, *R. mehelyi* and *R. hipposideros* (the latter with very low sample size) in Bulgaria, where they occur in sympatry. We recorded calls directly onto a laptop computer and used a specially designed analysis algorithm to achieve high and accurate frequency resolution. After silent periods, individuals 'tuned in' to their RFs always from lower frequencies, corroborating the recent finding that frequencies below RF might be used for auditory feedback control as well. *Rhinolophus blasii* could reliably be separated from its congeners by RF, while *R. mehelyi* overlapped strongly with both *R. euryale* and *R. hipposideros*. Only *R. blasii* showed sex and age differences in RF, albeit the overlap was large. Adult female *R. blasii* had higher RFs than both adult males and juvenile females. In *R. blasii*, RF was positively correlated with forearm length, body mass and body condition index; in *R. mehelyi* with body mass and body condition index. However, there was no correlation between RF and these body size parameters within a sex or age class for any of the species, suggesting that RF is not a reliable honest signal for intraspecific communication that would indicate the quality of a potential mate or competitor.

Key words: *Rhinolophus*, echolocation, resting frequency, body size, sex differences, honest signalling

INTRODUCTION

The key function of echolocation calls is to generate echoes that permit a bat to negotiate its three-dimensional environment and, for many bats, to find food (e.g., Schnitzler *et al*., 2003). However, echolocation signals are available to any animal that can hear them; i.e., echolocation inevitably also has a communicative function. Bats eavesdrop on other bats (Barclay, 1982; Fenton, 2003) and often will able to determine from the echolocation calls alone whether the other bat is a conspecific or not (Leonard and Fenton, 1984; Balcombe and Fenton, 1988), with obvious implications for agonistic or mutualistic interactions. Several bat species have been found to show age differences (mainly first year versus older) or sex differences in echolocation

signals (Neuweiler *et al*., 1987; Suga *et al*., 1987; Jones *et al*., 1992; Jones and Ransome, 1993; Jones *et al*., 1993, 1994; Jones and Kokurewicz, 1994; Masters *et al*., 1995; Pearl and Fenton, 1996; Moss *et al*., 1997; Kazial *et al*., 2001; Russo *et al*., 2001). However, these differences are small and call parameters often overlap for the sex or age groups, i.e., perfect discrimination is not possible. Only one behavioural study examined and showed that the bats can tell the caller's sex from echolocation signals (Kazial and Masters, 2004), while the acoustic discrimination of age classes has not yet been tested experimentally to our knowledge.

Within sexes, echolocation parameters could theoretically convey information on the size or body condition (i.e., quality) of a bat and thereby serve as honest signals (Bradbury and Vehrencamp, 1998) for potential mates or competitors. So far, very limited support is available for such an honest signalling hypothesis (Jones and Rayner, 1991; Jones *et al*., 1993; Guillén *et al*., 2000). The hypothesis is rendered less likely by the fact that the structure of echolocation calls is varied greatly in response to the current task the bat is facing (Siemers *et al*., 2001; Schnitzler and Kalko, 2001). Therefore, quality related information would have to be very robust not to be masked by the task-specific variability.

In the present study, we choose European horseshoe bats (*Rhinolophus*) to investigate whether echolocation calls contain reliable information on species, sex, age class or quality (size and body condition) of the caller. Horseshoe bats are an especially suitable model system, because their calls provide a clearly definable and robust frequency parameter that can be assessed accurately both by the bats and the researchers. That is because horseshoe bats (Rhinolophidae) and their sister taxon roundleaf bats (Hipposideridae) posses a specialized echolocation system. They broadcast comparatively long echolocation calls at a high duty-cycle with a prominent constant-frequency (CF) part and initial and terminal frequency-modulated (FM) components. Most energy is contained in the second harmonic. Frequency and amplitude shifts modulated onto the echoes of the CF parts by fluttering insect wings are used for prey detection and evaluation (Schnitzler, 1983; Schnitzler and Ostwald, 1983; Bell and Fenton, 1984; Emde, 1988; Emde and Menne, 1989; Emde and Schnitzler, 1990). This 'fluttering target detection' ability enables horseshoe bats to find insects even on echo-cluttering vegetation background or the ground (e.g., Jones and Rayner, 1989; Siemers and Ivanova, 2004), where they are difficult to detect for bats using FM calls (Arlettaz *et al*., 2001; Siemers and Schnitzler, 2004).

In horseshoe and roundleaf bats, hair cells and neurons responsible for response to frequencies at and around CF are very sharply tuned and over-represented both on the basilar membrane and at higher levels of the hearing system, constituting the so-called 'acoustic fovea' (Schuller and Pollak, 1979; Ostwald, 1984; reviewed in Neuweiler, 1990). To keep the returning echoes within the acoustic fovea, the bats compensate for the Doppler shift induced by their own flight speed by lowering the emission frequency accordingly (Schnitzler, 1968; Trappe and Schnitzler, 1982; Habersetzer *et al*., 1984; Metzner *et al*., 2002). In contrast to flying bats, stationary individuals do not experience Doppler shifts and hence emit calls at the so called 'resting frequency' (RF), which is about 50–250 Hz higher than the so called 'reference frequency' that matches the tuning of their acoustic fovea and hearing system (*Rhinolophus ferrumequinum* — Schnitzler *et al*., 1976). Therefore RF is often used as an indirect measure of the acoustic fovea of an individual bat; i.e., as a kind of morphological

parameter relating to the bats' hearing system. The difference between RF and foveal 'reference frequency' could theoretically be species specific. Nevertheless, RF is a suitable parameter to address the questions on signalling of species identity, sex, age class or quality by echolocation calls in horseshoe bats, because it is RF that (stationary) bats broadcast and that other bats hear. Within individuals, call frequency is positively correlated with body temperature (*Pteronotus parnellii* — Huffman and Henson, 1993). Jones and Ransome (1993) found seasonal changes in RF for *R. ferrumequinum* that are in the directions expected from a positive relation between body temperature and call frequency.

In an interspecific comparison, RF is closely matched to size parameters of the bats' external sound emission and receiving organs, namely nostril distance or nose-leaf width (Möhres, 1954; Pye, 1988; Bogdanowicz, 1992; Robinson, 1996) and ear size or cochlear width (Obrist *et al*., 1993; Francis and Habersetzer, 1998; Zhao *et al*., 2003). It is likely that a strong selection pressure keeps up the close match between RF and morphological traits of the bats sound emission and hearing system, because any significant deviation would worsen the echolocation performance for mere physical reasons (Denny, 2004). Across species, there further is a clear negative correlation of RF with body size for both rhinolophids and hipposiderids (e.g., Heller and Helversen, 1989; Bogdanowicz, 1992; Francis and Habersetzer, 1998; Jones, 1999; Kingston *et al*., 2000; Kingston and Rossiter, 2004).

There is not necessarily such a strong link between RF and other body size or life history parameters that are not directly involved in the echolocation process. However, if RF reliably differed between sexes or scaled with body size within a species, bats might use it as an honest signal (Bradbury and Vehrencamp, 1998) to learn something about a calling conspecific (Jones, 1995). RF does show sex differences in only few of the rhinolophids and hipposiderids studied to date (Neuweiler *et al*., 1987; Jones *et al*., 1992, 1993, 1994; Francis and Habersetzer, 1998; Guillén *et al*., 2000). In both bat families, RF has been found to scale with size (Jones *et al*., 1993; Francis and Habersetzer, 1998; Guillén *et al*., 2000), body condition (Jones *et al*., 1994; Guillén *et al*., 2000) and age (Jones *et al*., 1992, 1993; Jones and Ransome, 1993; Russo *et al*., 2001).

We recorded echolocation calls of four species of horseshoe bat in Bulgaria: the little-studied Blasius' horseshoe bat (*R. blasii*), the Mehelyi horseshoe bat (*R. mehelyi*), the Mediterranean horseshoe bat (*R. euryale*) and the lesser horseshoe bat (*R. hipposideros*). Previous work on these species in other areas of their distributional range in Europe had yielded support for an age difference hypothesis in British *R. hipposideros* (Jones *et al*., 1992), Italian *R. euryale* and *R. mehelyi* (Russo *et al*., 2001) with juveniles having lower pitched calls than adults. Sex differences were found for *R. hipposideros* (females call at higher frequencies than males — Jones *et al*., 1992), but not for *R. euryale* and *R. mehelyi* (Heller and Helversen, 1989; Russo *et al*., 2001). RF correlated positively with forearm length in juvenile *R. euryale* and with both forearm length and body mass in juvenile *R. mehelyi* in Italy; no correlation was found in adults (Russo *et al*., 2001). As in these previous studies, we took recordings from handheld bats to obtain the individuals' RFs. Building on technological progress, we were able to recorded the calls directly onto a laptop computer, thereby avoiding even slight frequency shifts that might be caused by time expansion systems in connection with tape recorders (when tape speed is not absolutely stable — Heller and Helversen, 1989). We extracted RF with

an analysis algorithm that yields high and accurate frequency resolution, allowing for the exploration of intraindividual variation. We tested the following specific hypotheses:
(1) Call frequencies of the sympatric Bulgarian populations of horseshoe bats differ sufficiently so that bats (and researchers) could determine species identity from the echolocation calls (hypothesis 1);
(2) RF differs reliably between sexes or age classes (adults vs. juveniles) and therefore could deliver valuable information to conspecifics (hypothesis 2);
(3) RF correlates with body size parameters (forearm length, mass, body condition index) and can hence be used as an honest, quality-related signal in intraspecific communication (hypothesis 3).

Materials and Methods

Field Sites and Animals

Fieldwork was carried out in September 2001 in Bulgaria under licence of Bulgarian authorities (MEW: No 48-00-56/16.01.2001, No 53-00-4428/23.05.2001). Bats were captured with a harp trap (1 m × 1 m, 4 frames, nylon threads of 0.12 mm diameter; Palmeirim and Rodrigues, 1993) when emerging from their day roosts at dusk. We captured bats on the 9th and 15th of September at Aina Ini Cave (41°24'N, 25°30'E) in the eastern Rhodope Mountains, on the 25th of September at Orlowa Tschuka Cave (43°35'N, 25°57'E) and on the 26th of September at Zorovitza Cave (close to Orlowa Tschuka) in a limestone formation in north-eastern Bulgaria. The latter two caves are at about 5 km distance from each other and therefore the bats might change easily from Orlowa Tschuka to Zorovitza Cave and vice versa. We refer to bats from both caves as belonging to the northern population of the respective species. The Aina Ini bats are referred to as the southern population.

Bats were sexed by inspecting the genitalia, weighed (Soehnle Ultra 200; precision 0.1 g) and forearm length was taken (Hommel precision caliper; precision 0.1 mm). We calculated a body condition index (BCI) for every individual as BCI (g/mm) = mass/forearm length (Russo *et al*., 2001). Bats were classified as adult or juvenile (i.e., being born in the same year). We distinguished juveniles from adults according to the following criteria (Dietz and Helversen, 2004): greyish general appearance, less dense fur with more uniform colour of ventral and dorsal fur, sharp and pointed teeth, lower body mass, small or invisible nipples and pubic nipples in females and thinner penis in males. Repeated recaptures of ringed individuals with known age corroborated that these are valid criteria for determination of age class. The standard criterion of incomplete fusion of phalange epiphysis in juveniles (Stebbings, 1986) proved to be invalid that late in the year, but in many juveniles the finger joints were broader than in adults.

We present data from 89 *R. blasii* (30 adult ♀♀, 20 adult ♂♂, 17 juvenile ♀♀, 22 juvenile ♂♂), 69 *R. euryale* (23 adult ♀♀, 40 adult ♂♂, 4 juvenile ♀♀, 2 juvenile ♂♂), 39 *R. mehelyi* (21 adult ♀♀, 15 adult ♂♂, 1 juvenile ♀, 2 juvenile ♂♂) and 3 *R. hipposideros* (all juvenile ♀♀). The *R. hipposideros* were disregarded for the statistical analyses because of the small sample size. For the same reason, the juvenile *R. euryale* and *R. mehelyi* were excluded from intraspecific comparisons of sexes and parameters of juveniles versus adults were only tested for *R. blasii*.

Sound Recording and Analysis

The bats were held in the hand about 1 m from the recording microphone. Echolocation calls were picked up with a custom built ultrasonic microphone (flat frequency response (± 3dB) between 30–120 kHz — Lehrstuhl Tierphysiologie Tübingen), A/D converted with custom built hard and software (Lehrstuhl Tierphysiologie Tübingen and PCTape, Menne BioMed) at a sampling rate of 480 kHz with 16 bit depth (additional 8-times oversampling for digital anti-aliasing filtering). The resulting digital signal was fed into a laptop computer via the USB-port and stored on the hard disk. The sound analysis package Cool Edit 2000 (Syntrillium Software Corporation) was used to evaluate the recordings and to select six calls with good signal-to-noise ratio per individual bat, wherever possible. We used an analysis window starting about 5 ms before the onset of the call and terminating 5 ms after its end. For each call, the frequency with most energy was computed in MATLAB 6.1 (The MathWorks, Inc.) with a custom written routine (K. Beedholm; available upon request). This routine performed an FFT (no additional time windowing function) on the entire signal contained in each wav-file, zero-padded up to next integer power of 2. In a frequency range ± 0.5 kHz around the peak energy frequency the chirp Z transform with 128 points was used to increase the resolution to 7.5 Hz. Furthermore a Lagrange interpolation polynomium was used on

the three points around the central peak of the chirp transform. The final estimate was then read from the peak of this 2nd degree polynomial. We verified with manual readings from time averaged spectra in Cool Edit that the frequency with highest energy over the whole signal reflected always the CF portion of the *Rhinolophus* calls. In tests with different pure CF signals, the analysis output was always correct with 0.1 Hz reading accuracy (difference between generated and measured CF frequency: 0.0 ± 0.0 Hz; $\bar{x}$ ± SD; n = 10). When testing horseshoe bat like FM-CF-FM signals, the difference between the generated CF frequency and the output of our analysis algorithm was 0.2 ± 0.1 Hz (n = 10). Therefore, within the frequency span where the Lagrange interpolation was made ($\Delta f < 7.5$ Hz), the presence of the FM components did influence the reading of the analysis algorithm, but to a very negligible degree. We further tested this issue by comparing the output for entire real bat signals and for the manually selected, isolated CF portion of these signals. The difference averaged 1.9 ± 2.4 Hz (n = 10). We ran the analysis algorithm over the entire signal instead of somewhat arbitrarily a priori defining a CF portion of the signal and cutting it out. The tests of the analysis algorithm gave us confidence that we were able to find the frequency with most energy over the entire signal, i.e., the CF frequency, with an accuracy considerably higher than 10 Hz, thereby improving resolution by at least a factor of 40 in comparison to most previous studies on the relation of CF to body size parameters.

Statistics

To assess intraindividual variation, we calculated the standard deviation (SD), standard error of the mean (SE) and the range of CF for every individual bat. For the subsequent analysis of sex, age class or size differences in call frequency, individuals were represented by their mean CF frequency (1° order mean) as an estimate of their individual RF. As some of the sub-datasets deviated significantly from a normal distribution (Kolmogorov-Smirnov test using Lilliefors adaptation; $P < 0.05$), we used nonparametric test throughout. For post-hoc pairwise comparisons, we used a procedure for nonparametric datasets with unequal numbers of data in each group following Zar (1999: 224, equations 11.26 and 11.27 after Dunn, 1964; Hollander and Wolfe, 1973: 125). For other multiple testing situations (Tables 2 and 3; Fig. 3), we followed Neuhauser (2004) in presenting individual P-values in combination with the summary P-value from the truncated product method (TPM p). TPM p's were calculated using a program (tpm.exe) provided at ftp://statgen.ncsu.edu/pub/zaykin/tpm (see Zaykin *et al*., 2002) to test whether any of the tests with $P < 0.05$ in the table are indeed significant (Neuhauser, 2004). In order to facilitate comparison of our descriptive statistics with published data, we give mean and standard deviation in addition to medians, minimum and maximum. Analyses were performed with Excel 2000 (Microsoft), Systat 10 (SPSS) and Jump 4 (SAS).

Results

Interspecific Differences

The handheld bats of all four species of horseshoe bat emitted FM-CF-FM calls with prominent CF components (Fig. 1). The four horseshoe bat species were well separated when plotting the individual RF (individual mean CF) against forearm

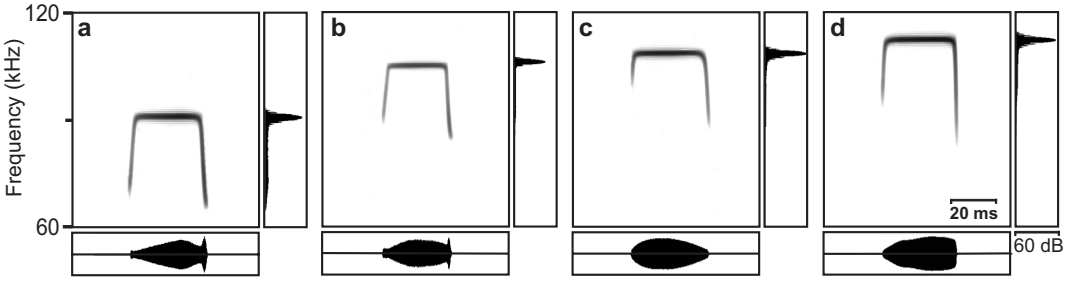

Fig. 1. Representative echolocation calls emited by handheld horseshoe bats from the species *R. blasii* (a — adult ♂), *R. euryale* (b — adult ♂), *R. mehelyi* (c — adult ♂) and *R. hipposideros* (d — juvenile ♀). Calls are depicted as spectrograms with amplitude waveform below (linear scaling) and averaged power spectrum on the right

length (Fig. 2). RF differed highly significantly between *R. blasii*, *R. euryale* and *R. mehelyi* (Kruskal-Wallis test and pairwise post-hoc comparisons — Table 1), albeit overlap between *R. euryale* and *R. mehelyi* was very high (Fig. 2). Likewise, RF of *R. mehelyi* and *R. hipposideros* overlapped; the latter being represented with a very small sample size only. *Rhinolophus mehelyi* had significantly larger forearms, higher masses and BCIs than both *R. blasii* and *R. euryale*, whereas the latter two differed only in forearm length and mass (Table 1).

Intraindividual Variation

Within individuals, the CF values we measured varied in a range from 31 Hz to 2.04 kHz (for individual standard errors see Figs. 2 and 3). The mean standard deviation of CF within an individual was 85 Hz for *R. blasii* (n = 89), 101 Hz for *R. euryale* (n = 69) and 93 Hz for *R. mehelyi* (n = 39). The larger variations in CF within an individual generally occurred after the handheld bats had ceased echolocation for some time (> 1 s) and then started calling again. In that situation, the bats would typically emit several calls with steadily increasing CF until reaching a constant frequency with a lower degree of variation (Fig. 4). The estimate of an individual's RF is likely to be especially reliable when the intraindividual variation of measured CF values is low. Therefore, we also analysed subsets of our data that included only bats with low intraindividual CF variation (either standard deviation ≤ 100 Hz or ≤ 50 Hz) when exploring possible correlations between RF and body size (Table 2). However, the fit was better and the significance

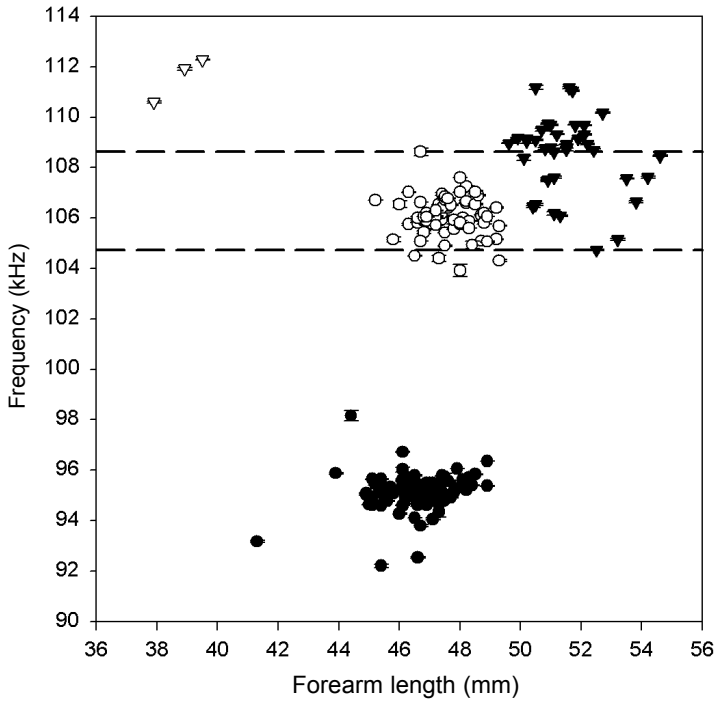

FIG. 2. Mean ± SE of the constant frequency (CF) component as an estimate for the individual resting frequencies (RF) are plotted against forearm length for *R. blasii* (●, n = 89), *R. euryale* (○, n = 69), *R. mehelyi* (▼, n = 39), and *R. hipposideros* (▽, n = 3), from Bulgarian populations. The dashed lines indicate the range of frequency overlap between *R. euryale* and *R. mehelyi*

TABLE 1. Interspecific differences in resting frequencies (RF), size parameters and body condition index (BCI) for three *Rhinolophus* spp. Pairwise post-hoc comparison following Zar (1999: 224)

Taxon	*n*	RF (kHz)			Forearm (mm)			Mass (g)			BCI (g/mm)		
		$\bar{x}$ ± SD	median	min–max	$\bar{x}$ ± SD	median	min–max	$\bar{x}$ ± SD	median	min–max	$\bar{x}$ ± SD	median	min–max
blasii	89	95.15 ± 0.74	95.22	92.21–98.16	46.6 ± 1.2	46.7	41.3–48.9	11.1 ± 1.2	10.9	7.9–13.7	0.24 ± 0.02	0.24	0.18–0.29
euryale	69	106.04 ± 0.78	106.00	103.92–108.63	47.6 ± 0.9	47.5	45.2–49.3	11.6 ± 1.2	11.4	9.2–15.5	0.24 ± 0.03	0.24	0.20–0.32
mehelyi	39	108.48 ± 1.52	108.80	104.74–111.18	51.5 ± 1.2	51.1	49.6–54.6	18.9 ± 3.8	20.4	11.8–24.9	0.37 ± 0.08	0.40	0.23–0.50
Kruskal-Wallis test comparing all three species													
		$H_2 = 160.6$	$P < 0.001$		$H_2 = 113.76$	$P < 0.001$		$H_2 = 89.28$	$P < 0.001$		$H_2 = 73.78$	$P < 0.001$	
Pairwise post-hoc comparison													
blasii – euryale		$P < 0.001$			$P < 0.001$			$P < 0.05$			ns		
blasii – mehelyi		$P < 0.001$			$P < 0.001$			$P < 0.001$			$P < 0.001$		
euryale – mehelyi		$P < 0.001$			$P < 0.001$			$P < 0.001$			$P < 0.001$		

was greater in the low-variation subsets as compared to the entire dataset in only one case (*R. blasii*: RF versus forearm; compare Fig. 3 and Table 2).

Intraspecific Variation

Within species, we found significant sex differences and age class differences in *R. blasii*, with adult females having higher RFs than both adult males and juvenile females (Fig. 3, Tables 3 and 4), but means differed by less than 1 kHz and overlap was large. The adult females ($n = 30$) had larger forearms, masses and BCIs (Mann-Whitney *U*-test), than the adult males ($n = 20$; $U_{forearm} = 524$, $U_{mass} = 564$, $U_{BCI} = 533$, all $P < 0.001$) and than the juvenile females ($n = 17$; $U_{forearm} = 379$, $P < 0.01$; $U_{mass} = 482$, $P < 0.001$; $U_{BCI} = 479$, $P < 0.001$). Accordingly, we found a positive correlation between RF and the three body size parameters in *R. blasii* when pooling all sex and age classes (Fig. 3, Table 2). Within the sex and age classes, we did not find any significant correlation between RF and the three body size parameters (Spearman rank correlation, *P*-level ranging from 0.14 to 0.86).

In *R. euryale*, we found sex differences in RF for the adult bats when analyzing the data from bats captured in northern and in southern Bulgaria separately (Table 3). Northern females were lower in RF than northern males, whereas southern females' RFs were higher than the respective males'. Within sexes, northern and southern adult *R. euryale* ($n = 48$ and 15, respectively) differed in RF (Tables 3 and 4); but with both sexes pooled, both medians (Mann-Whitney *U*-test, $U = 357$, $P = 0.96$) and distributions (Kolmogorov-Smirnov two sample test, $P = 0.89$) of RF did not differ between northern and southern adult *R. euryale*. The northern females had longer forearms than males ($n = 15$ and 33, respectively; Mann-Whitney *U*-test, $U = 355$,

$P < 0.05$) and the southern females had larger masses and BCIs than males ($n = 8$ and 7, respectively; Mann-Whitney U-test, $U_{mass} = 54$, $U_{BCI} = 55$, both $P < 0.01$). When pooling the adult *R. euryale* data from both northern and southern Bulgaria, the sex difference (23 ♀♀, 40 ♂♂) in RF (Table 3), mass ($U = 533.5$, $P = 0.29$) and BCI ($U = 502.5$, $P = 0.54$) was no longer evident. In the pooled dataset, females still had slightly larger forearms than males ($U = 607$, $P < 0.05$). There was a weak, non-significant

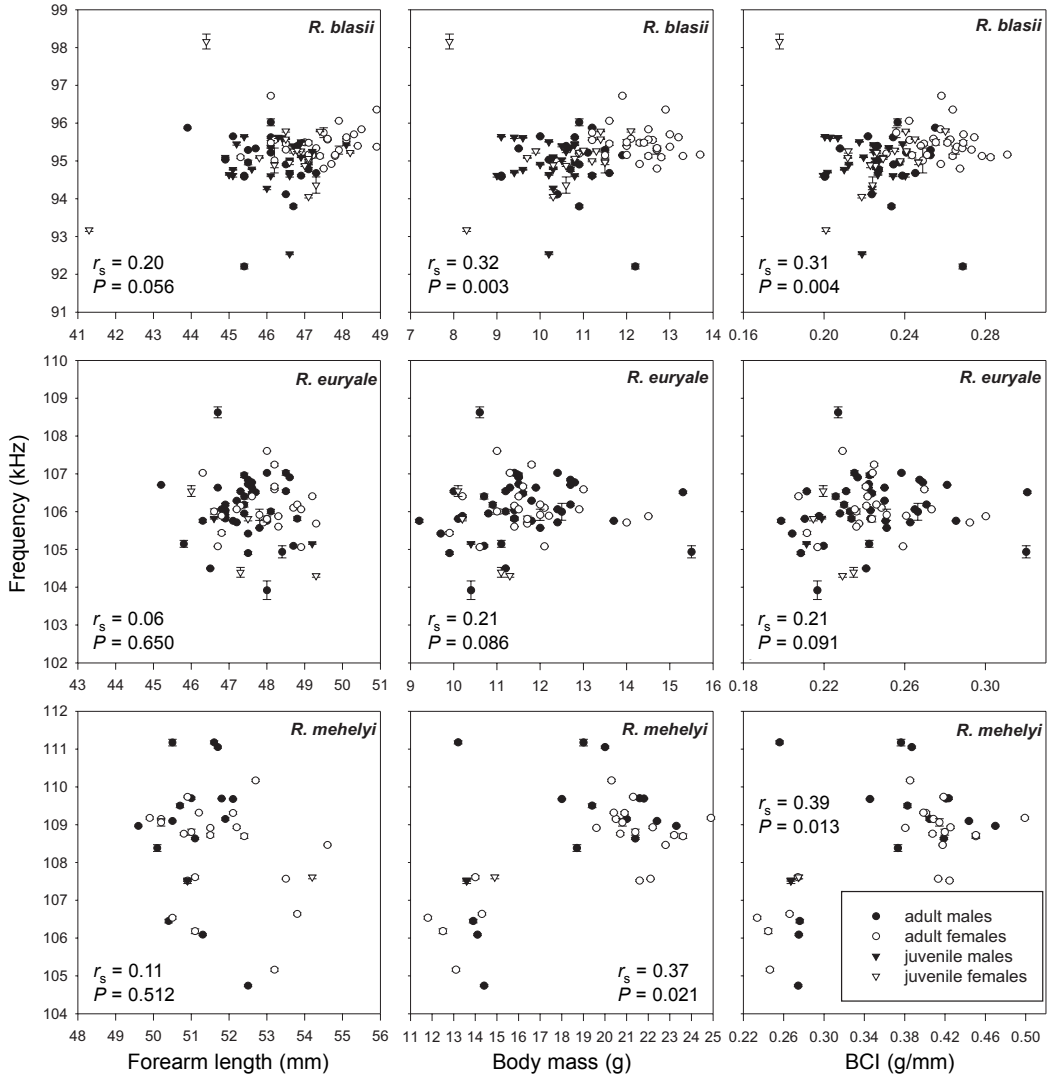

FIG. 3. Mean ± SE of the constant frequency (CF) component as an estimate for the individual resting frequencies (RF) are plotted against forearm length, body mass and the body condition index (BCI) for *R. blasii* ($n = 89$), *R. euryale* ($n = 69$) and *R. mehelyi* ($n = 39$) from Bulgarian populations. Sex and age groups are marked with different symbols (see legend). All frequency axes span 8 kHz and all forearm axes 8 mm; the absolute values are adjusted to show the entire range covered by the respective species. Note that the mass axes span 7 g and the BCI axes 0.15 g/mm for both *R. blasii* and *R. euryale* and double these values for *R. mehelyi*. In each panel, results from a Spearman rank correlation of RF on the respective body size parameter are given.

For all test results in this figure TPM P-value (truncated product method; see methods section) < 0.001

TABLE 2. Relation between RF and size parameters for each of the three *Rhinolophus* spp. for subsets of the data. Spearman rank correlations of RF with the subset with SD(CF) ≤ 100 Hz and the subset with SD(CF) ≤ 50 Hz are given; for correlations using the entire datasets see Fig. 3. The subset with SD(CF) ≤ 50 Hz was not analyzed for *R. mehelyi* because of too small sample size (n = 5). For all test results in this table TPM P-value < 0.01

Species	≤ 100 Hz							≤ 50 Hz						
	n	Forearm (mm)		Mass (g)		BCI (g/mm)		n	Forearm (mm)		Mass (g)		BCI (g/mm)	
		r_s	P	r_s	P	r_s	P		r_s	P	r_s	P	r_s	P
blasii	69	0.20	ns	0.34	0.004	0.35	0.003	42	0.38	0.013	0.29	0.059	0.24	ns
euryale	48	-0.01	ns	0.24	ns	0.22	ns	29	-0.09	ns	0.20	ns	0.19	ns
mehelyi	26	-0.02	ns	0.14	ns	0.18	ns	–	–	–	–	–	–	–

trend for a positive correlation between RF and both mass and BCI for *R. euryale* when data were pooled over all sex and age classes (Fig. 3, Table 2). Within the sex classes and geographical regions, we did not find any significant correlation between RF and the three body size parameters (Spearman rank correlation, P-level from 0.18 to 0.92).

For *R. mehelyi*, there was no difference in RF between northern and southern adult females nor males (Table 4), nor between all (32 northern and 4 southern) adult bats (Mann-Whitney U-test, U = 81, P = 0.39). Accordingly, we pooled the data from both geographical regions. We found no sex differences for the pooled data set in RF (Table 3) nor in the three body size parameters (Mann-Whitney U-test, $U_{forearm}$ = 191, P = 0.28; U_{mass} = 183, P = 0.41; U_{BCI} = 175, P = 0.57). In the pooled *R. mehelyi* data set, RF was unrelated to forearm length, but we found a positive correlation between RF and mass and BCI, respectively (Fig. 3, Table 2). Within the sex classes, there was no significant correlation between RF and the three body size parameters (Spearman rank correlation, P-level within 0.10–0.63).

DISCUSSION

Species Differences (Hypothesis 1)

Rhinolophus blasii can be separated from their three sympatric congeners just by the CF of their calls, supporting hypothesis 1. Likewise, confusion with the fifth European horseshoe bat species, *R. ferrumequinum*, should not be possible. RFs of *R. ferrumequinum* from southeast Europe range from 77 to 81 kHz (Heller and Helversen, 1989), while the lowest *R. blasii* RF we measured was at 92.2 kHz. Both bats and bat researchers might make use of the CF information to reliably identify *R. blasii*. Although *R. euryale* and *R. mehelyi* differ significantly in RF, the overlap between these two species is so large that, in contradiction to hypothesis 1, a reliable species identification based on CF is not even feasible for resting bats. The risk of misclassification of these two species in acoustic surveys has been highlighted earlier by Russo *et al*. (2001) for Italy. For flying bats, the picture will be blurred further both by the bats' Doppler shift compensation and the Doppler shifts caused by the bats' velocity relative to the recording microphone (or to the listening bat). A (fast) flying horseshoe bat at 7 m/s will lower its emission frequency to 96% of its RF. If the bat is flying straight towards a microphone, this microphone will record a Doppler shifted CF at 102% of the emission frequency. If the bat is flying straight away from a microphone, the recorded CF will be at 98% of the emission frequency. If we assume 7 m/s as the maximum flight speed (cf. Schnitzler, 1973; Aldridge, 1986; Langeheinecke, 2000), the

recorded CF of a flying horseshoe bat will be anywhere between RF and 6% below it. A correction of the recorded CFs will only be possibly when flight speed and direction relative to the microphone are known from, e.g., stereo videogrammetry (e.g., Holderied *et al*., 2005). Where this is not feasible, the 6% deviation translates into a maximal deviation of RF and the recorded CF of 5 to 6 kHz for the *Rhinolophus* species under study here (2 kHz, if the bat has been seen flying straight towards the microphone).

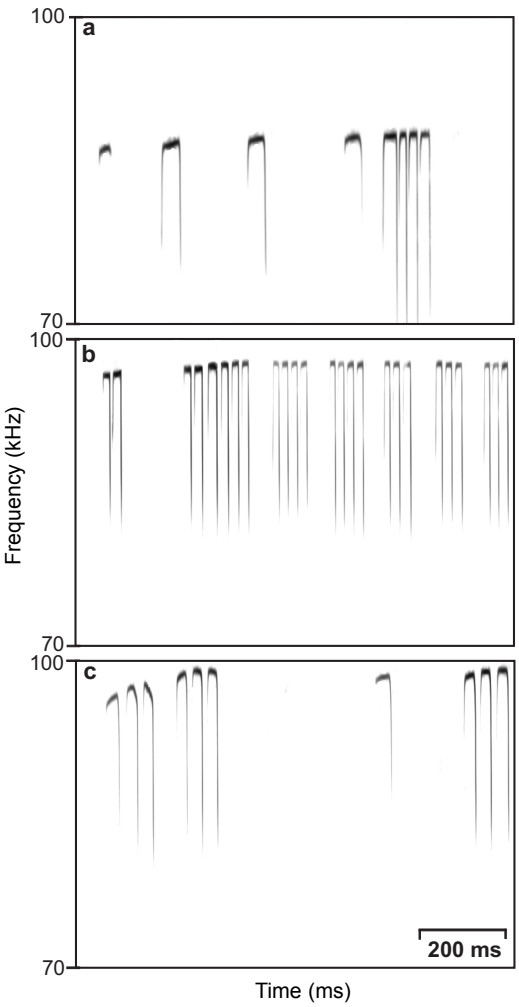

FIG. 4. Call sequences from handheld bats that resumed emitting echolocation calls after at least 1 s of silence. It takes several calls until the bats reach their individual RF. The sequences shown are the most extreme cases chosen to clearly exemplify this phenomenon. *R. blasii* (a — juvenile ♀), *R. euryale* (b — adult ♂), *R. mehelyi* (c — adult ♂)

It is interesting to note that the two similar sized species *R. blasii* and *R. euryale* differ markedly in RF, while *R. mehelyi*, that uses nearly the same frequency band as *R. euryale*, differs from the former two in size. *Rhinolophus mehelyi* RF likewise overlaps strongly with RF of the smallest horseshoe bats species in Europe, *R. hipposideros* (Heller and Helversen, 1989; this study). Further data on foraging and mating behaviour will be necessary to explore whether these patterns might reflect ecological segregation and resource partitioning or mate recognition (Heller and Helversen, 1989; Kingston and Rossiter, 2004). *Rhinolophus mehelyi* are at least locally considerably less abundant in our study area in southern Bulgaria as compared to the study area in northern Bulgaria (Benda *et al*., 2003; Ivanova and Gueorguieva, 2004). If CF of one species was influenced by other species that cover a similar or neighbouring frequency band (Heller and Helversen, 1989; Kingston *et al*., 2001; Kingston and Rossiter, 2004), one might expect that *R. euryale* use a larger frequency band or higher CFs in southern Bulgaria (where *R. mehelyi* are rare); provided the populations were sufficiently separated genetically to allow a CF shift to establish. However, we did not find any evidence for such acoustic character displacement.

The RFs we measured for Bulgarian *R. euryale* match well with those from Greek populations (Heller and Helversen, 1989), but are on average 2 to 3 kHz higher than mean values for French and Italian populations (Schnitzler, 1968; Heller and Helversen, 1989; Russo *et al*., 2001). Hence, the clinal variation of *R. euryale* RF over Europe shows an increase from West to East and therefore is in an opposite direction to the clinal variation known from

TABLE 3. Intraspecific differences in RF related to sex, age class or geographic area for three species of *Rhinolophus*. The last column gives results derived from the Mann-Whitney *U*-test. Further statistics in Table 4. TPM *P*-values for all test results for *R. blasii* and *R. eurvale* from both tables equal to 0.019 and 0.015, respectively

Age or geographic group	♀♀				♂♂				♂♂ versus ♀♀	
	n	x̄ ± SD	median	min–max	*n*	x̄ ± SD	median	min–max	*U*	*P*
					Rhinolophus blasii					
Adult	30	95.46 ± 0.41	95.43	94.80–96.72	20	94.89 ± 0.84	95.00	92.21–96.02	439	0.006
Juvenile	17	95.17 ± 1.0	95.08	93.18–98.16	22	94.95 ± 0.67	95.06	92.54–95.64	204	ns
					Rhinolophus euryale					
North Bulgaria	15	105.83 ± 0.41	105.88	105.06–106.41	33	106.21 ± 0.84	106.29	103.92–108.63	146	0.024
South Bulgaria	8	106.63 ± 0.62	106.63	105.89–107.60	7	105.56 ± 0.43	105.76	104.90–106.00	53	0.004
All adult animals pooled	23	106.11 ± 0.62	106.06	105.06–107.60	40	106.10 ± 0.82	106.05	103.92–108.63	447	ns
					Rhinolophus mehelyi					
Adult	21	108.31 ± 1.29	108.76	105.16–110.17	15	108.90 ± 1.87	109.15	104.74–111.18	113	ns

R. ferrumequinum (reviewed in Heller and Helversen, 1989).

Intraindividual Variation

The intraindividual variations found are extremely small compared with the absolute centre frequencies. The resolution of the frequency determination algorithm proved to be far beyond what is needed to resolve the intraindividual variation actually found. Thus, we do place some confidence in the actual physical presence of the variations we measured. For most bats, the intraindividual variation in CF was below 300 Hz, i.e., in the range known from other studies (Jones *et al*., 1992; Jones and Ransome, 1993; Guillén *et al*., 2000). Some individuals showed larger variations in CF. These were for the most cases individuals that had been silent for some time and resumed echolocation during our recordings. In all three *Rhinolophus* species, the first calls after such a silent period showed low CFs, that gradually 'tuned in' to the individuals' RFs. As the 'tuning in' usually happened within one second or less, we consider changes in body temperature (compare Huffman and Henson, 1993) to be an unlikely explanation for this phenomenon. Schuller and Suga (1976) reported for stationary *R. ferrumequinum* that the CF of the first call after a silent period was below RF and decreased with increasing duration of the silent period. These bats as well as those in our study always tuned into RF from below, possibly yielding additional support for Metzner *et al*.'s (2002) recent finding that frequencies not only above, but also below RF are important for auditory feedback control of the CF component. Obviously, the case we present does not involve Doppler compensation. While this 'tuning in' phenomenon might be interesting for understanding CF echolocation systems, it blurs the measurement accuracy of an

TABLE 4. Significance (Mann-Whitney *U*-test) of intraspecific differences in RF related to age class or geographic area for three species of *Rhinolophus*. Sample sizes as in Table 3

Species	♀♀		♂♂	
	U	*P*	*U*	*P*
R. blasii		adult versus juvenile		
	354	0.028	211	ns
R. euryale		North versus South		
	17	0.006	184	0.015
R. mehelyi		North versus South		
	31	ns	11	ns

individual's RF. We therefore had excluded individuals with high CF variation from part of the analyses (Table 2), what, however, did not produce markedly different results.

Sex Differences and Age Class Differences (Hypothesis 2)

The direction of *R. blasii* sex differences in RF coincides with those of other congeners (♀♀ higher than ♂♂ — Neuweiler *et al*., 1987; Jones *et al*., 1992). However, overlap in *R. blasii* was so large that a reliable determination of sex from echolocation calls will not be possible in most cases. In contrast, RF bands in *R. rouxii* are almost separated for the two sexes (Neuweiler *et al*., 1987), presumably allowing the bats to effectively recognize potential mates or competitors by listening to echolocation calls of stationary conspecifics. The sex differences we found in *R. euryale* when analysing northern and southern populations separately will deserve further study, as sample sizes were low and especially as the males in one location had higher RFs while females had higher RFs in the other (but see Guillén *et al*., 2000 for another example of geographically reversed sex differences in RF). From skull measurements on Bulgarian *R. euryale*, Popov and Ivanova (2002) reported a poorly developed sex difference (males having slightly larger skulls) and low, albeit present, geographic variability within the country.

We could analyse age differences only for *R. blasii* because of too limited sample sizes for the other species. Call frequency of adult *R. blasii* was higher than in juveniles, as reported for the other four European *Rhinolophus* species: *R. hipposideros* (Jones *et al*., 1992), *R. ferrumequinum* (Jones and Ransome, 1993), *R. euryale* and *R. mehelyi* (Russo *et al*., 2001). While overlap between juvenile and adult RFs is large in *R. hipposideros* (within sexes; Jones *et al*., 1992), *R. ferrumequinum* (Jones and Ransome, 1993) as well as in our *R. blasii* dataset, Russo *et al*. (2001) found little or no overlap between adult and juvenile *R. euryale* and *R. mehelyi*, suggesting that bats could use RF to reliably recognize the age class of a calling conspecific when hanging stationary. A likely explanation for the discrepancy between Russo *et al.*'s (2001) and our results is that recordings by Russo *et al.* (2001) were made earlier in the reproductive season (July–beginning of August) while ours were made in September. Juveniles undergo dramatic morphological and physiological changes over the season, and this also applies to their vocal tract and related features, such as call frequency. It might thus well be that RF can be used for age class recognition in the first weeks after young bats become volant. Final evidence for this age recognition hypothesis and its potential use in a social context will have to come from behavioural experiments. In summary, in contrast to some previous studies as detailed above, our data do not show a clear vocal dimorphism for sex or age classes and therefore do not strongly support hypothesis 2.

Correlation of RF and Body Size within Species: An Honest Signal (Hypothesis 3)?

In accordance with Russo *et al.*'s (2001) data on adult *R. euryale* from Italy, we did

not find any significant correlation between body size parameters and RF in *R. euryale*. While Russo *et al*. (2001) did not find any correlation for adult *R. mehelyi* either, we found RF to increase with mass and BCI in *R. mehelyi*. All seven *R. mehelyi* with RFs below 107 kHz were adult bats with low body mass and low BCI, but average forearm lengths. However, the maximum frequency differences found equate to an increase in wavelength of 0.2 mm, which will have a negligible effect on target resolution. It thus seems unlikely that foraging efficiency is mediated by differences in RF. Rather, the relationship between CF and body condition may be indicative of age-related decreases in foraging success as RF has been found to decrease in old bats, especially those over 10 years of age (Jones and Ransome, 1993).

The positive correlation between RF and body size in *R. blasii* was largely caused by the sex differences and by the age class related differences within females. In both cases, the large adult females had the highest RFs. Likewise, adult female *R. hipposideros* are larger than males and have higher RFs (Jones *et al*., 1992). This constitutes an interesting contrast to the clear negative correlation between RF and body size for the interspecific comparison in both bat families, which in turn can be explained by larger bats having larger vocal tracts and cavities and hence lower resonance frequencies (Pye, 1979). *Hipposideros ruber* on the other hand does follow the within-families trend in that (at most locations) males produce higher CFs and are slightly smaller (Guillén *et al*., 2000). The pattern is complicated further by several species with sex differences in RF, but not in body size (Neuweiler *et al*., 1987; Jones *et al*., 1994; Francis and Habersetzer, 1998), and clearly deserves further study.

Within sex and age groups, we never found clear correlations between RF and body size. For juveniles, sample sizes were only large enough in *R. blasii*. While Russo *et al*. (2001) showed an age or growth related increase of RF in juvenile *R. euryale* and *R. mehelyi* at the end of July and in the first days of August, our *R. blasii* data from September suggest that these effects vanish quickly within a bat's first 6 months of life. So far, the only rhinolophid or hipposiderid species that was found to show a clear correlation of RF with body size within sexes for adult bats is *Asellia tridens* (Jones *et al*., 1993). Guillén *et al*. (2000) report a correlation of body condition and RF within sexes for *Hipposideros ruber*. The lack of a clear correlation within sex classes in all other species studied (*R. rouxii*, *R. ferrumequinum*, *R. mehelyi*, *R. euryale*, *R. blasii* and *R. hipposideros* — Neuweiler *et al*., 1987; Jones *et al*., 1992; Jones and Ransome, 1993; Russo *et al*., 2001, and this study) indicates that RF is not especially suited as an honest signal that would reliably indicate the size or quality of a potential mate or competitor, thereby not supporting hypothesis 3. It is astonishing that RF remains rather constant even where large size differences are present. This might indicate that other selection factors keep it close to an 'optimal frequency' for the given species (e.g., Heller and Helversen, 1989; Guillén *et al*., 2000; Kingston and Rossiter, 2004). Further behavioural and ecological studies will be needed to resolve the importance of call frequency for communication and resource partitioning in horseshoe bats.

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