



Divergent echolocation call frequencies in insular rhinolophids (Chiroptera): a case of character displacement?

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ABSTRACT

Aims Because rhinolophids have been hypothesized to use echolocation call frequency to recognize conspecifics, sympatric species calling at similar frequencies should be subject to acoustic character displacement, i.e. a drift in frequency values to minimize the risk of misidentification of conspecifics. However, it has been proposed that insufficient geographical separation between populations in sympatry and allopatry may counter the establishment of frequency differences by character displacement. Here we tested the hypothesis that insular populations should exhibit acoustic divergence, and this should be revealed by comparing call frequencies with those observed in mainland, allopatric populations of conspecifics. We also tested whether the evolutionary pressure towards acoustic divergence should be especially strong at sites where rhinolophid species emitting similar call frequencies roost together in order to minimize interspecific frequency overlap.

Location Sardinia and southern Italy (Campania, Lazio, Abruzzo).

Methods Time-expanded echolocation calls and body size were recorded from Sardinian populations of *Rhinolophus mehelyi* Matschie, *Rhinolophus hipposideros* (Bechstein) and *Rhinolophus euryale* Blasius. Both call frequencies and forearm length of insular *R. hipposideros* and *R. euryale* were compared with those of populations from mainland areas of Italy where *R. mehelyi* is absent, to explore the hypothesis that the presence of the latter species (which calls at frequency values intermediate between the other two) may determine acoustic divergence in the other species. For Sardinian *R. mehelyi* and *R. euryale*, we also carried out intraspecific comparisons of call frequencies between bats from monospecific colonies and those from mixed colonies.

Results As hypothesized, Sardinian *R. hipposideros* and *R. euryale* called at frequencies higher and lower, respectively, than in the peninsula. In this way, overlap with *R. mehelyi* is avoided. Body size showed no difference between insular and peninsular populations, i.e. frequency differences are not a by-product of difference in body size determined by insularity. Frequency values in Sardinian *R. euryale* from monospecific colonies did not differ from those of bats roosting together with *R. mehelyi*. However, *R. mehelyi* showed frequency values significantly higher when associated with *R. euryale*, possibly to minimize the risk of species misrecognition.

Main conclusions At least under geographical isolation, character displacement may be a causal mechanism for shifts in call frequency of sympatric rhinolophids. Species recognition and facilitation of intraspecific communication (with possible implications for mate recognition) constitute the best candidate factors for the phenomenon we observed.

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Keywords

Allopatry, bats, character displacement, cultural transmission, geographical separation, islands, Mediterranean, resource partitioning, sympatry.

INTRODUCTION

Recent molecular studies have changed our view of bat evolution profoundly, highlighting the paraphyletic nature of microbats and providing evidence for the inclusion of rhinolophoid bats along with Old World fruit bats within the Yinpterochiroptera group (Springer *et al.*, 2001; Teeling *et al.*, 2002, 2005).

Such bats, including horseshoe bats (Rhinolophidae), have evolved a peculiar, sophisticated system of echolocation (Jones & Teeling, 2006). Typically, they produce calls made of a long constant-frequency (CF) component preceded and ended by brief frequency-modulated components (Schnitzler, 1968). Calls are produced with a high duty cycle (Fenton, 1994) and concentrate most energy in the second harmonic. Horseshoe bat echolocation is best suited to searching for moving prey in cluttered habitats such as forests, and offers high performance for the detection and classification of targets (Neuweiler, 1989; Schnitzler *et al.*, 2004; Jones & Teeling, 2006).

Each individual is most sensitive to a unique frequency value (the 'acoustic fovea'; Schuller & Pollak, 1979) which is close to the CF component emitted when stationary. Because of Doppler shifts, echolocation during flight leads to an apparent increase in frequency as perceived by a moving bat. To exactly match the level of maximal auditory sensitivity, the bat compensates for this shift by lowering the emitted frequency of the call (Schnitzler, 1968; Schuller & Pollak, 1979). Because of Doppler shift compensation, comparisons carried out to explore both intra- and interspecific differences best focus on frequencies of the maximum energy of pulses emitted by stationary bats – so-called 'resting frequencies', hereafter abbreviated as RF (e.g. Jones *et al.*, 1992; Russo *et al.*, 2001; Siemers *et al.*, 2005). By concentrating on RF, individual variation caused by flight speed is controlled for.

Social selection has been addressed as an important candidate causal factor for acoustic divergence among bat species. Heller & Helversen (1989) first highlighted a non-random evenly spaced call frequency distribution in a species-rich assemblage of syntopic rhinolophids. Two main hypotheses were put forward to explain this outcome: (1) differences between species might allow them to specialize in eating different sizes of prey; (2) alternatively, bats used call frequency for species recognition, and the observed pattern was linked with partitioning of communication channels. In fact, the frequency differences observed across rhinolophid species are sufficiently large to corroborate the hypothesis that bats employ RF to tell their own species apart from the others occurring in sympatry (Siemers *et al.*, 2005). RF values would

reflect a trade-off between orientation and detection tasks (related to foraging habitat structure and prey type) and recognition of conspecifics. Acoustic divergence in cryptic hipposiderids (another family of rhinolophoid bats) has been regarded as the consequence of social selection for a separate communication channel allowed for by the strict correspondence between signal features and receptor systems determined by the highly specialized echolocation system of rhinolophoids (Kingston *et al.*, 2001; Thabah *et al.*, 2006). Among vespertilionids, Jones & Barlow (2004) state that facilitation of intraspecific communication is the most parsimonious hypothesis for the acoustic divergence observed between cryptic species.

The maintenance of a 'private bandwidth' for communication may have crucial implications for social interactions and sexual behaviour, such as, for example, allowing bats to recognize groups of individuals or attract mates of the right species, with major expected consequences for survival and reproductive success. Reproductive character displacement results from the adverse consequences of misrecognizing sexual partners, selecting for greater differentiation of communication systems in areas of sympatry than in areas of allopatry. It seems to be operating in several animal groups relying on acoustic communication for mate attraction, such as insects (Marshall & Cooley, 2000; Shaw, 2000) and amphibians (Gerhardt, 1994; Gerlinde & Gerhardt, 2003); as largely vocal mammals, bats may also be subject to it.

Horseshoe bats communicate by a diverse repertoire of social calls (Andrews & Andrews, 2003; Ma *et al.*, 2006) that are mainly context-specific: they are emitted only in some circumstances and convey specific messages. Conversely, echolocation calls are constantly emitted by bats, and so may act as unambiguous badges of species identity: they can be an efficient, convenient and reliable means of signalling species status to conspecifics.

As mentioned, an alternative explanation for acoustic divergence in sympatric rhinolophids (Heller & Helversen, 1989) is that RF differences between sympatric species allow them to specialize in eating different sizes of prey. Echoes are strongest when the wavelength of sound is equal to or shorter than the wing length of the insect being ensonified (Houston *et al.*, 2004). When wavelength exceeds the wing length of the prey, echo reflectivity falls rapidly as frequency increases in the so-called 'Rayleigh scattering' region (Pye, 1993; Houston *et al.*, 2004). The wavelength of sound gives an estimate of the size of prey below which echo strength begins to fall off rapidly. By using a higher call frequency, a bat may therefore have calls better adapted for the detection of small prey, and call divergence may play a role in resource partitioning among

sympatric taxa. Acoustic divergence would then represent a case of ecological character displacement (Schluter, 2000; p. S5), i.e. 'the process of phenotypic evolution in a species generated or maintained by resource competition with one or more coexisting species'. In vespertilionid bats, morphological character displacement has been described as a way to achieve food niche partitioning (see, for example, divergence in jaw shape and size in sympatric *Myotis auricolus* and *Myotis evotis*; Gannon & Rácz, 2006).

Finally, differences in echolocation call frequencies might also reflect exploitation of different foraging habitats, and match differences in wing morphology (Jones & Barlow, 2004; Jacobs *et al.*, 2007). Apparent character displacement may be mimicked by local variation imposed by environmental differences between sites of sympatry and allopatry (Schluter, 2000). In this case, the RF shift would be determined by environmental factors rather than by the presence of species calling at similar frequencies.

One difficulty in testing for frequency displacement is that several factors influencing RF, such as geographical differences (e.g. Heller & Helversen, 1989; Vaughan *et al.*, 1997), body size, sex or age (Neuweiler *et al.*, 1987; Jones *et al.*, 1992, 1993; Jones & Ransome, 1993; Russo *et al.*, 2001; Siemers *et al.*, 2005), may possibly simulate, reduce or mask it.

Theoretically, if RF plays a role in either species recognition (i.e. facilitation of intraspecific communication) or niche partitioning, RF divergence in rhinolophid species broadcasting similar calls should be more pronounced in sympatry than in allopatry. Therefore, a comparison of RF between sympatric and allopatric populations would help understand its adaptive value, but insufficient genetic (i.e. geographical) separation between populations in sympatry and allopatry may counter the establishment of frequency differences by character displacement (Siemers *et al.*, 2005). This is especially true for species as mobile as bats, which may disperse, or migrate, over long distances (Hutterer *et al.*, 2005), leading to potentially frequent contact between sympatric and allopatric populations. Based on this consideration, we propose that frequency divergence is therefore likely to evolve most readily in geographically isolated populations, such as those on islands.

We tested whether, under the effective isolation occurring on an island, observed RF values are in agreement with the hypothesis of acoustic character displacement in sympatric rhinolophids. Sardinia proved an ideal study area, being isolated (c. 180 km distant) from the mainland coast, and inhabited by three species – *Rhinolophus euryale* Blasius, *Rhinolophus mehelyi* Matschie and *Rhinolophus hipposideros* (Bechstein) – whose neighbouring frequency ranges are potentially exposed to character displacement.

Italian *R. mehelyi* show a peculiar geographical range, being practically absent on the peninsula but numerous and widespread in Sardinia (e.g. Mucedda, 1994; Mucedda *et al.*, 1995; Mitchell-Jones *et al.*, 1999). RF ranges of Sardinian *R. mehelyi* partly overlap with those of *R. euryale* recorded in peninsular Italy (Russo *et al.*, 2001). When both juveniles and adults are considered (the former calling at significantly lower

frequencies in both species), calls range between 99 and 105 kHz and 100 and 109 kHz, respectively, in peninsular *R. euryale* and Sardinian *R. mehelyi*. However, it is unknown whether this overlap also occurs between sympatric populations.

Rhinolophus hipposideros typically calls at frequencies higher than *R. mehelyi*, but values are reported to largely overlap (Heller & Helversen, 1989; Siemers *et al.*, 2005). In peninsular Italy, calls emitted by *R. hipposideros* in flight (i.e. affected by Doppler shift compensation) peak at 107–114 kHz (Russo & Jones, 2002).

In this study, we predict that because of the occurrence of a large *R. mehelyi* population (numerically dominant over the other rhinolophid species) in geographical isolation, RF in sympatric *R. euryale* will shift towards lower values, and the opposite trend (i.e. a shift towards higher frequencies) will occur in *R. hipposideros*. To assess the direction and magnitude of frequency shifts, we compared Sardinian *R. euryale* and *R. hipposideros* with populations in peninsular Italy inhabiting areas where *R. mehelyi* is absent. We also estimated the magnitude of RF differences between sympatric species to discuss whether they best corroborate the hypothesis of ecological, rather than social, character displacement.

Because roosts are the main sites for social interactions (e.g. Kunz, 1982), besides focusing the analysis on a macrogeographical scale (i.e. Sardinia vs. the mainland) we also explored the hypothesis that RF shifts might occur locally, at caves where different species emitting similar call frequencies roost together. In such cases, more frequent interspecific interactions might promote local acoustic divergence. We did that for both Sardinian *R. euryale* and *R. mehelyi*, which form mixed nursery colonies in the south of Sardinia; the prediction could not be applied to *R. hipposideros*, which has different ecological requirements and roosts apart in the breeding season (it mainly selects buildings instead of caves for reproduction; Schober & Grimmberger, 1997).

MATERIALS AND METHODS

In Sardinia we sampled bats at several roosts (*R. euryale*, $n = 4$; *R. hipposideros*, $n = 9$; *R. mehelyi*, $n = 5$) in May–October 2005, 2006. Sites were located between latitude 39°11' N–40°49' N, longitude 8°30' E–8°49' E. *Rhinolophus euryale* and *R. hipposideros* were also sampled in peninsular Italy (Campania, Lazio and Abruzzo regions, at sites lying between latitude 41°17' N–42°33' N, longitude 11°44' E–14°29' E). Bats were either caught during evening emergence with 2.5 × 6 m and 2.5 × 12 m mist nets (50 denier, 38-mm mesh) or harp traps, or captured with a hand net inside the roost. All captures were carried out under licence from the Italian authorities.

We restricted our analysis to adult bats, distinguished from juveniles by trans-illuminating wings (juveniles show cartilage epiphyseal plates in finger bones and more tapered finger joints; Anthony, 1988). For each bat, we recorded species, sex and forearm length (hereafter abbreviated as FAL) to the nearest 0.1 mm.

We recorded a total of 228 adult bats, as follows: 53 *R. euryale* in Sardinia (22 females, 31 males) and 51 in peninsular Italy (31 females, 20 males); 48 *R. hipposideros* in Sardinia (23 females, 25 males) and 26 in peninsular Italy (21 females, 5 males); and 51 *R. mehelyi* in Sardinia (26 females, 25 males).

Bat echolocation calls were sampled as 10× time-expanded signals with a Pettersson D980 bat detector (Pettersson Elektronik AB, Uppsala, Sweden) positioned at *c.* 30 cm from hand-held bats and recorded with a Sony TCD D8 DAT recorder. The detector sampling frequency was 350 kHz.

Signals were subsequently re-digitized with the software BatSound 3.31 (Pettersson Elektronik AB) at a sampling frequency of 44.1 kHz (16 bits per sample).

We generated call spectrograms by applying a 512-point FFT (fast Fourier transform), 98% overlap, with a Hamming window. A 112-Hz frequency resolution was obtained for spectrograms and power spectra.

When rhinolophids start echolocating, initial calls may show transient, lower frequency values before reaching the final RF level (Siemers *et al.*, 2005). Such calls were not considered for analysis. When calls were emitted in 'batches', only one call per group was chosen (Russo *et al.*, 2001). We analysed calls whose CF portion lasted at least 10 ms. Among such calls, 10 per bat were selected at random. The CF component was then selected to generate the power spectrum, from which RF (frequency of maximum energy in kHz) was measured. Mean RF values were used for analysis.

In order to determine the wing length of insect prey below which the echo strength becomes increasingly weak, we calculated wavelength (in mm) as $1000 \times (\text{speed of sound in air at } 25^\circ\text{C and } 80\% \text{ relative humidity}) / \text{mean call frequency (Hz)}$. The speed of sound = 347.65 m s^{-1} . This allowed us to estimate whether any differences in call frequency might influence resource partitioning by allowing the different species to specialize in the detection of different sizes of prey.

A two-way analysis of variance (general linear model) was applied to test for differences in mean RF and forearm length; sex and geographical area (Sardinia, peninsular Italy) were entered as the main factors. Analysis of variance was also used to compare RFs within species between bats roosting together with other rhinolophids (syntopy) and those roosting in monospecific colonies. This analysis was applied to Sardinian *R. euryale* and *R. mehelyi*. Because bats were randomly sampled from a large number of roosts, any influence of microgeographical or colony effects (Guillén *et al.*, 2000) probably affected the data set at random so we expected no significant influence of these factors on the comparison between populations we carried out. Previous work in the same areas (Russo *et al.*, 2001) had shown that body condition index ($100 \times \text{body mass/forearm length}$) had no influence on RF, so this variable was not included in the analyses. Data sets departing from a normal distribution of residuals were normalized by a log-transformation. The normality of the distribution was checked with a Ryan–Joiner test. Significance was set at $P = 0.05$. Analyses were carried out with Minitab® rel. 13.1 (Minitab Inc., State College, PA, USA).

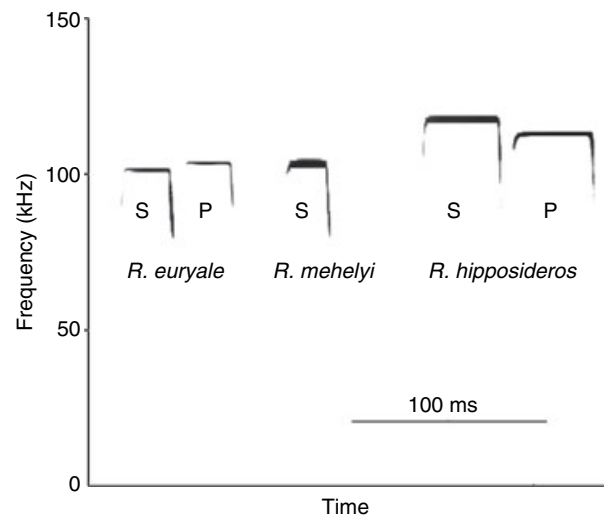


Figure 1 Spectrograms of second (dominant) harmonics of typical echolocation calls in rhinolophids from Sardinia (S) and peninsular Italy (P). Note that frequency value of the constant-frequency portion in Sardinian *Rhinolophus euryale* Blasius is lower than in peninsular bats, whereas the opposite is observed in *Rhinolophus hipposideros* (Bechstein). FFT size = 512 points.

RESULTS

Sardinian vs. peninsular bats

Sardinian and peninsular *R. euryale* differed significantly in their RF, whereas no influence of sex on this parameter was detected (Figs 1 & 2, Table 1). As predicted, Sardinian *R. euryale* called at lower values (RF = 101.38 ± 0.38 kHz) than peninsular bats (RF = 103.84 ± 0.56 kHz). In both areas, females had larger FAL than males, but the size of Sardinian bats did not differ from that of peninsular ones. Moreover, the interaction between sex and area was not significant (Table 1).

Both sex and geographical area influenced RF in *R. hipposideros* (Figs 1 & 2, Table 2). As predicted, Sardinian bats called at higher values. Females had greater RF values than did males, and Sardinian females called with mean RF values of *c.* 4 kHz greater than did peninsular bats; for males, this difference was on average *c.* 3 kHz (Table 2). In both areas, FAL was sexually dimorphic (females were larger than males); however, no geographical difference in FAL was observed, i.e. bats from Sardinia and peninsular Italy did not differ in size. In this case too the interaction between sex and area was not significant (Table 2).

Rhinolophus mehelyi showed no sexual dimorphism in either RF (females = 106.94 ± 1.28 kHz; males = 107.02 ± 1.58 kHz; $F_{1,49} = 0.04$, n.s.; Fig. 1) or body size (forearm length females = 50.8 ± 0.9 mm; males = 51.3 ± 1.2 mm, $F_{1,49} = 2.9$, n.s.).

Wavelength differences among species, and within each species between Sardinian and peninsular bats, were negligible, suggesting that the minimum size of prey detectable by the echolocation calls of all species should be broadly similar. In *R. mehelyi* the wavelength was 3.25 mm, whereas in Sardinian

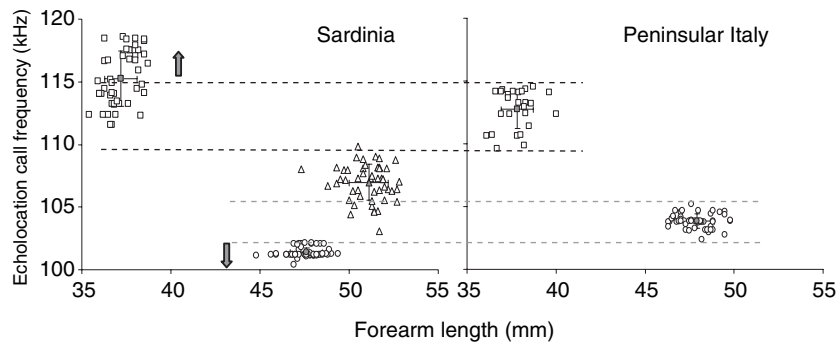


Figure 2 Scatterplot of echolocation call frequencies (recorded from stationary bats) plotted against forearm length in *Rhinolophus euryale* Blasius (circles), *Rhinolophus mehelyi* Matschie (triangles) and *Rhinolophus hipposideros* (Bechstein) (squares) from Sardinian and peninsular (Italian) populations. Empty symbols are single data points, filled symbols show means, error bars show standard deviations. Dashed grey lines show upper and lower values of frequency ranges in peninsular bats. Note how the ranges of peninsular bats, and especially that of *R. euryale*, overlap with that of Sardinian *R. mehelyi*. Arrows illustrate the expected direction of acoustic character displacement for insular *R. euryale* and *R. hipposideros*.

Table 1 Descriptive statistics and two-way ANOVA (general linear model; GLM) details for frequency of highest energy (a, b) and forearm length (c, d) in *Rhinolophus euryale* Blasius.

(a) Frequency of highest energy (kHz)				
	<i>n</i>	Mean	SD	
Sardinia	53	101.38	0.38	
Peninsular Italy	51	103.84	0.56	
(b) GLM ANOVA				
Factor	d.f.	Adj. MS	<i>F</i>	<i>P</i>
Sex	1	0.00	0.27	n.s.
Area	1	0.00	673.21	< 0.001
Sex × area	1	0.00	3.06	n.s.
Error	100	0.00		
(c) Forearm length (mm)				
	<i>n</i>	Mean	S.D.	
Sardinia				
Males	31	47.3	0.84	
Females	22	47.9	0.83	
Peninsula				
Males	20	47.5	0.72	
Females	31	48.1	1.01	
(d) GLM ANOVA				
Factor	d.f.	Adj. MS	<i>F</i>	<i>P</i>
Sex	1	10.57	13.80	< 0.001
Area	1	0.81	1.04	n.s.
Sex × area	1	0.00	3.06	n.s.
Error	100	0.76		

Interactions between factors are indicated with a '×' sign. Populations from Sardinia and peninsular Italy were compared. SD = standard deviation, Adj. MS = adjusted mean squares; n.s. = not significant ($P > 0.05$). Statistical tests were carried out on log-transformed values of frequency of highest energy.

R. euryale and *R. hipposideros* it was 3.43 and 3.01 mm, respectively. For peninsular populations of the latter two species, the wavelength was 3.34 and 3.08 mm.

Table 2 Descriptive statistics and two-way ANOVA (general linear model; GLM) details for frequency of highest energy (a, b) and forearm length (c, d) in *Rhinolophus hipposideros* (Bechstein).

(a) Frequency of highest energy (kHz)				
	<i>n</i>	Mean	SD	
Sardinia				
Males	25	113.48	1.03	
Females	23	117.20	1.28	
Peninsula				
Males	5	110.61	0.66	
Females	21	113.26	1.24	
(b) GLM ANOVA				
Factor	d.f.	Adj. MS	<i>F</i>	<i>P</i>
Sex	1	0.00	91.07	< 0.001
Area	1	0.00	104.20	< 0.001
Sex × area			2.14	n.s.
Error	70	0.00		
(c) Forearm length (mm)				
	<i>n</i>	Mean	SD	
Sardinia				
Males	25	36.8	0.73	
Females	23	37.7	0.72	
Peninsula				
Males	5	37.5	0.89	
Females	21	37.9	0.95	
(d) GLM ANOVA				
Factor	d.f.	Adj. MS	<i>F</i>	<i>P</i>
Sex	1	5.70	8.78	< 0.001
Area	1	2.17	3.34	n.s.
Sex × area	1	1.1	1.68	n.s.
Error	70	0.65		

Interactions between factors are indicated with a '×' sign. Populations from Sardinia and peninsular Italy were compared. SD = standard deviation, Adj. MS = adjusted mean squares; n.s. = not significant ($P > 0.05$). Statistical tests were carried out on log-transformed values of frequency of highest energy.

Bat species roosting together vs. those roosting in monospecific colonies

We compared 33 *R. euryale* roosting in monospecific nursery colonies with 21 from mixed colonies with *R. mehelyi*. Because sex had no effect on RF values, we applied a one-way ANOVA, which showed that no difference occurred between the two roosting situations (RF = 101.4 ± 0.4 kHz and 101.3 ± 0.3 kHz, respectively, for bats roosting alone and those sharing roosts with *R. mehelyi*; $F_{1, 52} = 0.81$, n.s.).

When the same test was applied to compare *R. mehelyi* from monospecific and mixed colonies (46 and 5 bats, respectively), in the latter (where *R. euryale* was also present) RF was significantly higher (RF = 106.8 ± 1.3 kHz and 108.7 ± 0.9 kHz respectively; $F_{1, 49} = 8.98$, $P < 0.005$). As with *R. euryale*, in the final ANOVA model sex was removed since it had no influence on RF values.

DISCUSSION

The analysis focusing on a large geographical scale (Sardinia vs. peninsular Italy) showed that, at least under strong geographical isolation, call frequencies in *R. euryale* and *R. hipposideros* occurring in sympatry with *R. mehelyi* are more divergent than in regions where the latter is absent. When Sardinian bats roosting in syntopy were compared with those roosting in monospecific colonies, *R. euryale* showed no difference, whereas data on *R. mehelyi* roosting with *R. euryale*, albeit from a limited sample, suggested the occurrence of an upward frequency shift, as expected to minimize interspecific frequency overlap. Although correlative, the evidence we provide supports the hypothesis of character displacement (Losos, 2000) and this may explain the observed frequency divergence. The divergence might result from random effects (such as genetic drift; Jones & Barlow, 2004). However, the probability that calls from Sardinian and peninsular populations of *R. euryale* randomly showed the observed difference on a macrogeographical scale (RF Sardinia < RF peninsula) equals 0.33 (the remaining cases would have no difference between populations, or higher values in Sardinian bats). The same probability (0.33) holds for the random occurrence of *R. hipposideros* calling at higher frequencies in Sardinia. The co-occurrence of the above cases would have a probability of random occurrence of only $0.33 \times 0.33 = 0.11$. It is therefore likely that call divergence evolved in the directions observed through natural selection minimizing RF overlap among sympatric taxa. Ruling out chance as an explanation of the observed pattern is a main criterion supporting observational evidence for character displacement (Schluter, 2000).

Although echolocation call differences may relate to differences in body size (e.g. Heller & Helversen, 1989; Jones, 1999), this was not the case for the differences in echolocation call frequencies observed between peninsular and Sardinian populations of *R. euryale* and *R. hipposideros*, respectively, as demonstrated by the absence of significant differences in forearm length.

Trophic niche partitioning by differences in the ability to detect prey of different sizes is not a credible candidate causal mechanism for the observed acoustic divergence: our findings are most consistent with the hypothesis that the shift was driven by social, rather than ecological, character displacement (Schluter, 2000).

Frequency may importantly influence reflectivity in relation to target size only for large frequency differences (Houston *et al.*, 2004), which was clearly not the case with Sardinian and peninsular rhinolophids. Differences in wavelength, and consequently in the wing length of prey below which reflectivity falls rapidly, were negligible: the three species have calls with very similar wavelengths, and the frequency shift of insular relative to peninsular *R. euryale* and *R. hipposideros* is unlikely to have any effect on selection of target insects differing in size from those detected by *R. mehelyi*. We therefore reject the hypothesis that divergence of call frequency evolved to promote resource partitioning by prey size. The absence of correspondence between FAL and RF, as well as the lack of interaction between sex and area (Sardinia, peninsula) on FAL in both *R. hipposideros* and *R. euryale* suggest that no relationship exists between wing morphology and echolocation frequency differences in insular and peninsular populations of either species, a fact which would rule out the hypothesis that differences in RF reflect exploitation of different foraging habitats. Similarly, the foraging habitat hypothesis has also been refuted in a study addressing the reasons for deviation from allometry of echolocation frequency (RF higher than expected according to body size) in another rhinolophid, *Rhinolophus clivus* (Jacobs *et al.*, 2007). In that case too, the relatively high call frequency has been attributed to partitioning of frequency bands aimed to minimize ambiguity during social interactions.

In the cryptic pipistrelle species *Pipistrellus pipistrellus* (Schreber) and *Pipistrellus pygmaeus* (Leach) the major factor promoting acoustic divergence was associated with facilitating communication with conspecifics, rather than resource partitioning (Jones & Barlow, 2004). The two bats broadcast echolocation signals containing most energy on average at 45 and 55 kHz, respectively (e.g. Jones & Van Parijs, 1993). Experimental work demonstrated that differences in target strengths of midges and dungflies (major prey for *P. pygmaeus* and *P. pipistrellus*, respectively) ensouffied at the typical peak frequencies of these bats were too limited to contribute to resource partitioning: facilitation of intraspecific communication would best explain the evolution of acoustic divergence (Jones & Barlow, 2004). As in that case, the divergence in call frequency we found is most likely to have been driven by social character displacement, allowing each species to have a 'private bandwidth' for species recognition (Thabah *et al.*, 2006).

Why should Sardinian *R. euryale* call at frequencies lower than in the control area (peninsula)? According to our results, if frequencies emitted by adult *R. euryale* in Sardinia equalled those observed in peninsular Italy, the overlap with adult *R. mehelyi* would occur in the range 103–105 kHz (Fig. 2). The

overlap with juvenile *R. mehelyi* (calling at frequencies of 100–105 kHz; Russo *et al.*, 2001) would be even greater. The potential overlap between *R. hipposideros* and *R. mehelyi* is more limited. In fact, call frequencies of adult peninsular *R. hipposideros* and Sardinian *R. mehelyi* would overlap between 109 and 110 kHz (Fig. 2). However, the problem would mainly concern juvenile *R. hipposideros*, since at least during the very first months of life rhinolophids typically emit lower frequencies than adults (Jones *et al.*, 1992; Jones & Ransome, 1993).

Roosting bats may potentially detect and interpret cues other than acoustic ones (e.g. involving vision or olfaction) that are useful for species recognition. Moreover, at roosts where more rhinolophid species congregate, species recognition is also likely to be achieved through the complex vocal repertoire of social calls typical of these bats (for *R. ferrumequinum* see Andrews & Andrews, 2003; Ma *et al.*, 2006). However, echolocation calls may provide important information for species recognition at foraging sites or along commuting routes (Heller & Helversen, 1989). Similarity in habitat preferences increase the likelihood that bats from different species interact at foraging sites. In the case we considered, *R. mehelyi* and *R. euryale* often visit the same foraging habitats, mainly represented by forested areas (Russo *et al.*, 2002, 2005; Goiti *et al.*, 2003), and *R. hipposideros* also is a forest specialist (Bontadina *et al.*, 2002). The existence of an ultrasound-based mechanism of specific recognition in flying bats might also be of use when swarming around roost sites such as when preparing for hibernation, or for selection of common foraging ground. There is evidence that in *Rhinolophus ferrumequinum*, juveniles associate with their mothers at foraging grounds, and inherit maternal preferences for particular areas (Rossiter *et al.*, 2002). Recognition of conspecifics in flight appears to be an indispensable prerequisite for the occurrence of subtler mechanisms of individual, or kin, recognition, and most probably for correct identification of sexual partners.

Interestingly, some (albeit limited) overlap between call RF from sympatric *R. euryale* and *R. mehelyi* sharing the same roost is documented for Spain (Salsamendi *et al.*, 2005). Likewise, for Bulgarian populations of the three species considered in our study, Siemers *et al.* (2005) failed to find the divergence we observed. However, they recognize that gene flow between mainland populations that are often not isolated by physical barriers may limit divergence in call frequency. The influence of the extent of geographical separation on call frequency values is highlighted in studies on within-species frequency variation in relation to island size (Pedley, 2004): colonies that are not located far enough apart to prevent migration and movement of individuals do not show frequency differences. The importance of geographical isolation with respect to intraspecific vocal differences is also well known for birds. Shared vocalizations decline with increased distances among populations, because distance both reduces the chance of mutual learning and encourages the accumulation of local mistakes during song

learning (Avery & Oring, 1977; Ficken & Weise, 1984; Tracy & Baker, 1999; Tu & Severinghouse, 2004). In our case, the great distance of Sardinia from the mainland may have been sufficient for the establishment of the observed frequency differences, probably – as we propose – as a result of character displacement.

These considerations may also help explain the results of the within-species comparison made for *R. euryale* and *R. mehelyi*, respectively, in monospecific and mixed colonies. *Rhinolophus euryale* is mainly confined to the south of Sardinia, so we expect the gene flow to be higher there, countering the establishment of local RF differences. Consequently, RF values did not differ between pure and mixed colonies.

Our study also showed that RF was higher in *R. mehelyi* roosting together with *R. euryale* than in monospecific colonies. Although caution is advisable in interpreting this result due to the limited sample size of *R. mehelyi* from mixed colonies, the difference observed is in agreement with the hypothesis that RF may be locally different to minimize overlap between species (in this case with *R. euryale*). The greater geographical distance between pure and mixed *R. mehelyi* colonies might limit gene flow and consequently encourage the establishment of local RF variants which minimize interspecific overlap.

According to Schluter (2000), heritability of the modified character is another major factor to be considered in character displacement studies. A template determining call structure is certainly genetically determined (Jones & Ransome, 1993). However, cultural transmission, as well as genetic influences, may also favour divergence in call frequency between isolated populations. For example, Jones & Ransome (1993) showed that fine-scale variation in call frequency in *Rhinolophus ferrumequinum* was partly attributable to juveniles learning from their mothers. In captive *Hipposideros terasensis*, bats transplanted into new social groups seemed to shift RF to match that of existing colony members (Hiryu *et al.*, 2006).

In this study, *R. euryale* showed no vocal sexual dimorphism, whereas this clearly occurred in *R. hipposideros*. Both findings are in agreement with previous work (Jones *et al.*, 1992; Russo *et al.*, 2001; Salsamendi *et al.*, 2005; Siemers *et al.*, 2005). However, for Bulgarian *R. euryale* populations, Siemers *et al.* (2005) detected RF sexual dimorphism only when recordings from northern and southern Bulgaria were considered separately. Interestingly, the direction of dimorphism was opposite for the two areas, females calling at lower frequencies than males in the north and vice versa in the south. These differences vanished when the entire data set was pooled together for analysis.

Although our study represents a first attempt at testing acoustic displacement in insular rhinolophids, we are aware that more case studies on sympatric vs. allopatric (especially if separated by sufficient geographical barriers) populations are needed to obtain a general, large-scale picture of the role of character displacement as a factor influencing call frequency in bats.

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