

Asymmetric frequency shift in advertisement calls of sympatric frogs

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Abstract. Character displacement is commonly observed when species occur in secondary contact zones and traits related to resource competition or reproduction diverge in sympatry. However, few studies have considered the factors determining and delimiting the direction of character evolution in this context. We studied displacement in advertisement calls in two species of hyliid frogs from allopatric and sympatric populations, both of which call with similar frequencies but differ substantially in temporal parameters. We found asymmetrical character displacement in sympatry, as only *Scinax madeirae* (but not *S. fuscomarginatus*) repeatedly showed displacement. Instead of diverging in already existing differences in temporal characters, *S. madeirae* showed character displacement for frequency-related characters. We explored possible reasons for this specific pattern concerning the displaced characters and tested if socio-functional constraints in specific call parameters are responsible for the shift of only spectral parameters in that species. Finally, we argue that the simultaneous action of ecological and reproductive character displacement, or alternatively, a short-term behavioral response for the same reason (avoidance of hybridization) could explain the pattern. The present study identifies a set of new hypotheses that will stimulate future research on mechanisms of mate recognition and behavioral responses.

Keywords: ecological character displacement, mating call variation, reproductive character displacement, *Scinax fuscomarginatus*, *Scinax madeira*.

Introduction

Species that occur in both allopatry and sympatry (e.g., in secondary contact zones) are prime candidates to unravel mechanisms of adaptive character displacement (Dobzhansky, 1940; Brown and Wilson, 1956; Grant, 1972; Higgie, Chenoweth and Blows, 2000). On the one hand, ecological character displacement (ECD) is regularly seen in sympatric congeners and is usually attributed to selection caused by competition for limited ecological resources (Slatkin, 1980; Howard, 1993; Schluter, 2000; Pfennig and Pfennig, 2010). Reproductive character displacement (RCD), on the other hand, stems

from selection to lessen sexual interactions between reproductively competing species (reviewed in Pfennig and Pfennig, 2009, 2010), and can derive from reinforcement mechanisms through selection against hybrids, giving rise to pre-zygotic reproductive isolation mechanisms (Dobzhansky, 1937, 1940; Howard, 1993; Coyne and Orr, 2004). For instance, character displacement in anurans was demonstrated for an array of species (e.g. Blair, 1955, 1974; Littlejohn, 1965, 1999; Loftus-Hillis and Littlejohn, 1992; Gerhardt, 1994; Pfennig, 2000; Pfennig and Pfennig, 2005, 2010; Lemmon and Lemmon, 2010; Rice and Pfennig, 2010; Richards-Zawacki and Cummings, 2010). RCD regularly concerns traits related to mate finding and choice, such as male courtship signals (temporal call parameters: Fouquette, 1975; Lemmon, 2009; spectral call parameters: Höbel and Gerhardt, 2003), but other aspects of reproductive biology can also be affected (e.g., aggregation behavior of calling males: Pfennig and Stewart, 2011; use of different types of calling perches: Höbel and Gerhardt, 2003; or female preference: Márquez and Bosch, 1997). Mechanistically, RCD in male courtship signals can be caused by selection against signal

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interference (Gerhardt and Huber, 2002), or it can occur when male signal design responds to diverging female preferences that evolved to decrease the chance of heterospecific matings (Boul et al., 2007). Pfennig and Pfennig (2009) described how initial differences between traits of the competing species facilitate character displacement and can be emphasized in sympatry (see also fig. 9.8 in Littlejohn, 2001).

Whereas many recent studies of RCD have considered the role of divergence in acoustic signals in population divergence and speciation (Ptacek, 2000; Boughman, 2002; Hoskin et al., 2005; Funk et al., 2009), only a few address the factors delimiting and determining the direction of signal evolution (Wilkins, Seddon and Safran, 2013). The mechanisms of both ECD and RCD as well as their outcomes are thus still far from being well understood, and little is known about the generality of trends in character displacement in general (including RCD and ECD). For example, does a recognizable pattern exist in character displacement in replicated secondary contact zones? More specifically, is divergence uniformly seen in characters that are similar or those that are already different in the allopatric situation? In the present study, we compared male advertisement calls in several sympatric and allopatric populations of congeneric hyliid frogs (*Scinax madeira* and *S. fuscomarginatus*). We asked if there is character displacement of the male courtship signal in sympatry, and if so, whether there are asymmetries among call characters (i.e., different temporal and frequency components of the call; see Fouquette, 1975; Höbel and Gerhardt, 2003; Lemmon, 2009) in their responses to selection. Based on the finding that only frequency-related parameters are displaced in one of the two species in sympatry (i.e., *S. madeirae*; see Results), we tested the hypothesis that limits on acoustic divergence plays a role in call evolution of these species (Wilkins, Seddon and Safran, 2013). More specifically, we asked if social functions of the call (e.g. species or individual recognition: Davies, 1987; Ryan and

Rand, 1993; Bee and Gerhardt, 2001; Gerhardt and Huber, 2002; Gasser, Amézquita and Hödl, 2009; Bee et al., 2010) could limit the standing variation of these traits and thus constrain their evolvability in secondary contact zones.

Additionally, we conducted empirical tests for two hypotheses seeking to explain why only one species shows displacement. (i) First, we asked if the less abundant species would be more likely to diverge and thus examined relative abundances of both species at localities where they occur in sympatry (see review in Cooley, 2007). (ii) Second, we asked if *S. madeirae* in allopatric populations shows higher call variability (standing variation), which could be indicative of a predisposition to evolve (Slatkin, 1980; Pfennig and Pfennig, 2009). However, the tests remained inconclusive (see online Supplements 1-3).

Materials and methods

Study system

The species *Scinax fuscomarginatus* and *S. madeirae* (recently removed from the synonymy of *S. fuscomarginatus* by Brusquetti et al., 2014) are phylogenetically closely related and morphologically similar species that offers a unique framework to study evolution and speciation processes. *Scinax fuscomarginatus* has a wide distribution in open landscapes of South America, from the Venezuelan, Guianan and Suriname lowlands throughout north and northwest Brazil reaching the north of Argentina, while *S. madeirae* is more restricted, occupying eastern Amazonia (State of Rondônia, Brazil) and eastern Bolivia (Brusquetti et al., 2014). During an amphibian inventory using molecular, morphological, and bioacoustic data in Bolivia (Jansen et al., 2011) the existence of two main genetic lineages related to *S. fuscomarginatus* corresponding to *S. fuscomarginatus* and *S. madeirae* was verified (in Jansen et al., 2011 as *S. parkeri* and *S. cf. fuscomarginatus*, respectively). In Bolivia some populations are in sympatry where they are bioacoustically distinguishable (fig. 1). In this study we use the term “sympatric” for localities, where both species were heard at the same site (syntopic). As mentioned above, taxonomic status of those lineages was recently assessed by Brusquetti et al. (2014). Their molecular phylogenetic analysis shows the Bolivian sympatric populations of *S. fuscomarginatus* and *S. madeirae* in distant genetic subclades that suggests secondary contact (Stuart, Inger and Voris, 2006; Inger, Stuart and Iskandar, 2009; Brusquetti et al., 2014).

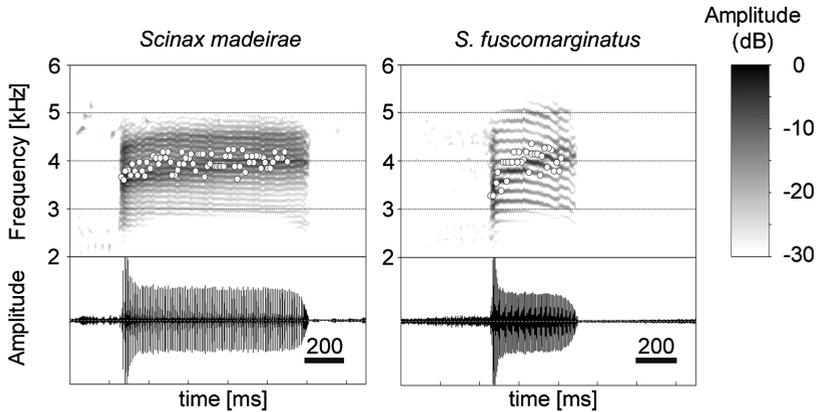


Figure 1. Example audiospectrogram (above; 1024 FFT size, 50% overlap, no filter) and waveform (below) of advertisement calls of sympatric *Scinax madeirae* (left; recorded at 26.6°; SVL 24 mm), and *S. fuscomarginatus* (right; recorded at 24.3°; SVL 22.5 mm) from site San Sebastián. White dots indicate dominant frequency.

Data collection and storage of acoustic data

Field work for data collection was conducted between 10 January and 15 February 2010 and between 13 January and 10 March 2012. In total, recordings from 20 sympatric and allopatric populations at 16 localities were analyzed in this study ($N = 12$ allopatric and $N = 4$ sympatric sites; fig. 2). We included a recording from Márquez et al. (2002) that was formerly assigned to *S. cf. fuscomarginatus*, and in our opinion (and as corroborated by our present analyses), is a call of a *S. fuscomarginatus* male; and a topotype call of *S. madeirae* from the type locality Porto Velho, Rondônia, Brazil from Brusquetti et al. (2014).

We obtained bioacoustic recordings on site between 1900 and 2400 h using a directional microphone (Sennheiser ME 80 microphone with M 66 power module) attached to a digital recorder (Olympus DM-550) with a sampling rate of 44 kHz and saved as uncompressed *.wav files. We measured air temperature to the nearest 0.1°C with a digital thermo-hygrometer hand-held close to the calling male after the recording. Only advertisement calls were analyzed, whereas sporadic, territorial, or fighting calls (see Toledo and Haddad, 2005) were not considered. For the present study we analyzed 1039 calls from 134 individual males – two to 14 calls from each individual (mean \pm SD = 8 ± 2 calls, depending on the quality of the recordings); most of the statistical analyses (see below) were based on mean values of the different call characters for each individual male. To guarantee the permanent storage and the access for public (Toledo, Tipp and Márquez, 2015), copies of the original recordings obtained for this study are deposited in the sound library Tierstimmenarchiv, Berlin (<http://www.tierstimmenarchiv.de/>). A list of the recordings and their according data, as well as library reference numbers can be found in the online Supplement 7.

Museum vouchers and tissue samples

We collected 130 calling males (one to 12 individuals per locality, not more than two at each capture site within the different sampling sites). At each capture site, frogs occurred

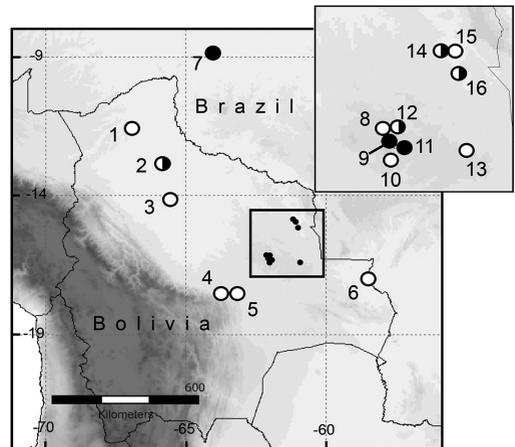
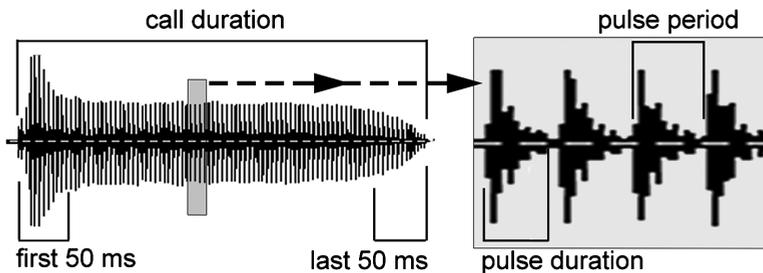


Figure 2. Sampling sites in Bolivia and Brazil. (1) Baracón, Pando, Bolivia (data collected by J. Moravec, 28 January 2006); (2) Los Lagos (Selvablu), Beni, Bolivia; (3) Estación Biológica Beni (EBB), Beni, Bolivia (data taken from Márquez et al. 2002); (4) Buenavista, Santa Cruz, Bolivia; (5) Isla Bonita, near Santa Cruz de la Sierra, Santa Cruz, Bolivia; (6) El Espinal, Santa Cruz, Bolivia; (7) Porto Velho, Rondônia, Brazil (data collected by F. Brusquetti, November 2009); (8) 9.4 km E of Concepcion, Santa Cruz, Bolivia; (9) Tucuman, Santa Cruz, Bolivia; (10) Concepción, Santa Cruz, Bolivia; (11) Inselberg, Santa Cruz, Bolivia; (12) Centro de Investigaciones Ecológicas Chiquitos, San Sebastián, Santa Cruz, Bolivia; (13) San Ignacio de Velasco, Santa Cruz, Bolivia; (14) Lagunitas, outpost of Hacienda Caparú, Santa Cruz, Bolivia; (15) Hacienda Caparú, Santa Cruz, Bolivia; (16) Campamento, Santa Cruz, Bolivia. White circles: *Scinax fuscomarginatus* populations; black circles: *Scinax madeirae*; black and white inner circles: sympatric populations.

Table 1. Abbreviations and definitions of the nine call traits used in this study.

Name	Abbreviation	Definition
Minimum frequency	MNF	Lower frequency bound of the vocalization
Maximum frequency	MXF	Upper frequency bound
Dominant frequency	DF	The frequency at which the maximum power is seen, taken for the entire call
Dominant frequency at the beginning of the call	DFB	Measured within the first 50 ms of each call
Dominant frequency at the end of the call	DFE	Measured within the last 50 ms
Instantaneous pulse rate	PR	Inverse of the interval between the beginning of pulse n to the beginning of pulse $n + 1$; measured at one pulse in the middle of the call
Pulse duration	PD	Smallest subunit of the call, measured from the beginning to the end of the pulse
Call duration	CD	Time from the beginning to the end of the vocalization
Instantaneous call rate	CR	Inverse of the interval between the beginning of call n to the beginning of call $n + 1$

**Figure 3.** Representation of temporal call variables measured for this study.

in aggregations of chorusing males with more than 200-500 individuals per chorus, and we believe removing individuals had no effect on the local population. Individual males were captured by hand, euthanized by intraperitoneal injection of 0.5 ml of a narcotic (T61[®], Intervet), stored in 70% ethanol. Measurements of snout-vent length (SVL) were taken to the nearest 0.1 mm under a dissecting microscope using digital calipers. From a subset of males, we preserved tissue samples (small pieces of muscle tissue) for DNA analysis in 96% ethanol (see Jansen et al., 2011 for molecular species identification). The online Supplement 4 shows the call characteristics and barcoding results for one remarkable specimen from the Lagunitas site, outpost of Hacienda Caparú (Santa Cruz) that showed intermediate vocalization behavior and may be a hybrid individual.

All procedures adhere to the legal requirements of Bolivia, and permissions for research (MMAyA-VMABCC-DGBAP No. 941) and export (SANASAG 10886) are available.

Statistical analyses

We analyzed advertisement calls with RAVEN PRO v 1.4 (Bioacoustics Research Program, 2011). Recordings were

resampled at a rate of 44 kHz and 16-bit resolution. Frequency information was obtained through Fast Fourier Transformation (FFT, width: 1024 points). Spectrograms were obtained using the Hanning window function with 1024 points resolution. We measured nine call parameters that are defined in table 1 (see also fig. 3): minimum frequency (MNF), maximum frequency (MXF), dominant frequency (DF), dominant frequency at the beginning of the call (DFB), dominant frequency at the end of the call (DFE), instantaneous pulse rate (PR), pulse duration (PD), call duration (CD), and instantaneous call rate (CR). To visualize audio-spectrograms and waveforms we used SEEWAVE R package (Sueur, Aubin and Simonis, 2008) (fig. 3). To avoid variations in MNF and MXF through changes in the overall signal intensity, the recording level and distance, as well as spectrogram parameters were standardized.

Ambient temperature and body size influence anuran call parameters (Zweifel, 1959, 1968). Temperature may even affect female preferences (Gerhardt, 1978), but those shifts in female preferences may or may not be paralleled by concomitant changes in male calls (Gerhardt and Mudry, 1980). In this study we present both original, uncorrected call data and data that were corrected for body size and temperature, because we consider both types of data to be important

sources of information. The original call data (frequency as well as temporal parameters) represent what females hear in their natural environment and are the stimuli to which they respond. Correction of the data, on the other hand, allows us to identify changes in call parameters independent of body size and temperature. Thus, the analyses described below (PCA, MANOVA, GLMM) were run twice, once with the original data (i.e., call variables as measured on site) and then with residuals of a preparatory MANCOVA to remove body size (SVL) and temperature effects on the calls. For the preparatory MANCOVA we used SVL and temperature as covariates and the nine z -transformed variables (to standardize scaling) as dependent variables and saved the resulting residuals. The preparatory MANCOVA confirmed highly significant effects of temperature ($F_{9,123} = 3.026$, $P = 0.003$) and size ($F_{9,123} = 11.176$, $P < 0.0005$). The results of separate, univariate ANCOVAs (as a step-down analysis after the MANCOVA) on influences of temperature and size on each call parameter (not shown in detail) revealed that all parameters were influenced by size ($P \leq 0.013$) except MNF ($P = 0.22$) and CR ($P = 0.79$). On the other hand, the effect of temperature was only significant in ($P = 0.03$), PD ($P = 0.05$), DFB ($P = 0.02$) and DFE ($P = 0.01$; all other parameters $P \geq 0.44$). In neither case were interaction terms of “species by temperature” or “species by body size” significant, suggesting that no slope heterogeneity occurred between species (results not shown).

To detect potential character displacement in sympatry we applied Principal Component Analyses (PCA), based on a rotated component matrix. In both cases we retained three PCs with eigenvalues > 1.0 (see Results). We then calculated MANOVAs using the three PCs as dependent variables (see Tobler et al., 2011 for an example), while including “species”, “sympatry/allopatry”, and their interaction term as factors. As random factors (in our case the factor “site”) cannot be included in MANOVA models (Langerhans, 2009), we also calculated separate generalized mixed models (GLMM, Tobler et al., 2011) for each PC while including “site” as a random effect to account for potential random variation between sampling sites. Again, “species”, “sympatry/allopatry”, and their interaction term were included as fixed factors. In these analyses we were mainly interested in the interaction term of “species \times sympatry/allopatry” which – if significant – would be indicative of character displacement in sympatry. Note that we were not expecting the significant difference in the main factor “sympatry/allopatry”, as a significant effect would indicate a general shift in call parameters between allopatric and sympatric sites in the same direction in both species. Also note that a significant interaction effect would only become apparent in these analyses if (a) only one species shows character displacement and repeatedly diverges in the same direction, or (b) if both species diverge but repeatedly diverge in opposing directions. In other words: this kind of analysis was suited to detect shared responses in RCD across replicated sympatric populations. All statistical analyses were conducted using SPSS 17.0.

Test for potential limits of call divergence

Different traits in anuran mating calls can have different functions in interactions between conspecifics and heterospecifics such as species recognition (Ryan and Rand, 1993; Gerhardt and Huber, 2002) or individual recognition (Gerhardt, 1991; Bee et al., 2001, 2010; Feng et al., 2009; Gasser, Amézquita, Hödl, 2009; e.g. “dear-enemy effect”: Davies, 1987; Bee and Gerhardt, 2001). These functions tend to be associated with “static traits”, which are traits with low intra-individual (within-male) variability. Such limits on standing variation, however, actually could explain why only some (in our study: spectral) traits are involved in the character displacement, whereas other (temporal) traits not. More specifically, we developed and tested the hypothesis that temporal parameters may function as cues for individual recognition, and thus, that the “static” nature (and thus, the limits on standing variation) of this socio-functional trait could impose constraints on the evolvability of those components of the courtship signal in secondary contact zones.

In order to test this hypothesis, we conducted an empirical test based on allopatric populations, asking if there are traits characterized by low within-male call variation. We used the coefficients of variation ($CV = SD \times 100/\text{mean}$) for each male and each analyzed call trait to characterize the variability of each trait (Gerhardt, 1991; Márquez and Eekhout, 2006; Pröhl et al., 2007; Käfer et al., 2012). The grand mean of *within-male* call [$CV_{wi} = SD \text{ (of } n \text{ calls of individual } m) \times 100/\text{mean (of } n \text{ calls of individual } m)$; Gerhardt, 1991; Castellano et al., 2002; Bee et al., 2010] was then calculated of each trait and each species. For subsequent comparisons *among males*, however, our data set of allopatric populations is yet too small and further sampling as well as behavioral experiments to test the function of certain cues for individual recognition will be needed.

Results

General characteristics of male advertisement calls

Original call data are presented in table 2 and example calls of both species visualized in fig. 1. Calls of *Scinax madeirae* were characterized by an average (mean \pm SD) call duration of 825 ms (± 126 ms; ranging from 558 to 1277 ms averaged over all localities; see table 2 for details regarding allopatry and sympatry), a pulse rate of 132 pulses s^{-1} (± 11 pulses s^{-1} ; ranging from 97 to 151 pulses s^{-1}), and a dominant frequency of about 4085 Hz (± 552 Hz; ranging

Table 2. Descriptive statistics [mean \pm SD (min-max)] of nine call traits of *Scinax madeirae* and *S. fuscomarginatus*. Shown are original data, not corrected for SVL and ambient temperature. Values were obtained by calculation of the mean, SD, minimum and maximum of all calls of all males (and not the mean of the mean values of each male).

	<i>Scinax madeirae</i>		<i>S. fuscomarginatus</i>	
	Allopatry	Sympatry	Allopatry	Sympatry
	115 calls <i>N</i> = 18 individuals	365 calls <i>N</i> = 43 individuals	338 calls <i>N</i> = 46 individuals	221 calls <i>N</i> = 27 individuals
MNF (Hz)	2055 \pm 419 (1012-2971)	2280 \pm 448 (1002-3392)	2222 \pm 422 (962-3315)	2026 \pm 455 (860-3171)
MXF (Hz)	5932 \pm 613 (4009-7875)	6195 \pm 771 (4505-8770)	5983 \pm 638 (4404-8018)	6199 \pm 835 (4943-10188)
DF (Hz)	3734 \pm 335 (3100-4608)	4197 \pm 561 (3144-5672)	4018 \pm 465 (2928-5211)	4105 \pm 456 (3359-5383)
DFB (Hz)	3624 \pm 270 (3100-4522)	3978 \pm 500 (3058-5578)	3879 \pm 494 (2713-5168)	3828 \pm 462 (3144-5383)
DFE (Hz)	4068 \pm 227 (3617-4651)	4420 \pm 385 (3445-5718)	4296 \pm 331 (3361-5211)	4246 \pm 404 (3359-5383)
PR (Hz)	130 \pm 5.6 (119-143)	132 \pm 11.8 (97-151)	213 \pm 15 (166-250)	217 \pm 19 (169-277)
PD (ms)	4.3 \pm 0.7 (3.0-6.4)	4.4 \pm 1.0 (2.0-7.6)	3.1 \pm 0.5 (1.3-4.5)	3.1 \pm 0.5 (1.6-4.7)
CD (ms)	845 \pm 73 (646-1091)	819 \pm 137 (558-1277)	385 \pm 72 (154-661)	379 \pm 70 (218-563)
CR (Hz)	0.48 \pm 0.30 (0.04-1.87)	0.40 \pm 0.20 (0.04-1.79)	0.57 \pm 0.47 (0.02-2.05)	0.56 \pm 0.50 (0.03-1.87)
SVL (mm)	24.3 \pm 1.1 (22.5-26.8)	22.5 \pm 1.8 (19.3-26.6)	21.9 \pm 1.2 (19.2-24.1)	21.8 \pm 1.1 (19.1-23.3)
Temperature ($^{\circ}$ C)	25.05 \pm 0.6 (24.5-25.8)	25.6 \pm 2.7 (18.6-29.2)	25.3 \pm 1.3 (22.0-27.2)	25.7 \pm 1.6 (22.4-28.5)

from 3100 to 5672 Hz) with a slightly lower beginning (3893 \pm 480 Hz; ranging from 3058 to 5578 Hz) and a slight increase in frequency toward the end of the call (4336 \pm 384 Hz; ranging from 3445 to 5719 Hz). In calls of *S. fuscomarginatus* mean call duration was only 383 ms (\pm 71 ms; ranging from 154 to 661 ms), and pulse rate was higher, at 215 pulses s^{-1} (\pm 17; ranging from 166 to 277 pulses s^{-1}). Similar to the former species, *S. fuscomarginatus* also had a mean dominant frequency of about 4050 Hz (\pm 463 Hz; ranging from 2928 to 5383 Hz), and again, the dominant frequency was slightly lower at the beginning (3859 \pm 481 Hz; ranging from 2713 to 5383 Hz), and increased at the end (4276 \pm 362 Hz; ranging from 3361 to 5383 Hz).

Character displacement. Original call data

Qualitatively, frequency-related (spectral) call characteristics appeared to be more dissimilar between species when comparing the sympatric to the allopatric situation (table 2; fig. 4), while temporal call parameters invariably differed between species in both situations (table 2; online Supplement 5). Specifically, *S. madeirae* calls had lower frequencies than *S. fuscomarginatus* in allopatry, but rather than accentuating these differences in sympatry, frequency-related parameters shifted dramatically towards higher frequencies in *S. madeirae* (see for example fig. 4c, d). The only exception was MXF, which was shifted towards higher frequencies in both species in sympatry. SVL also differed

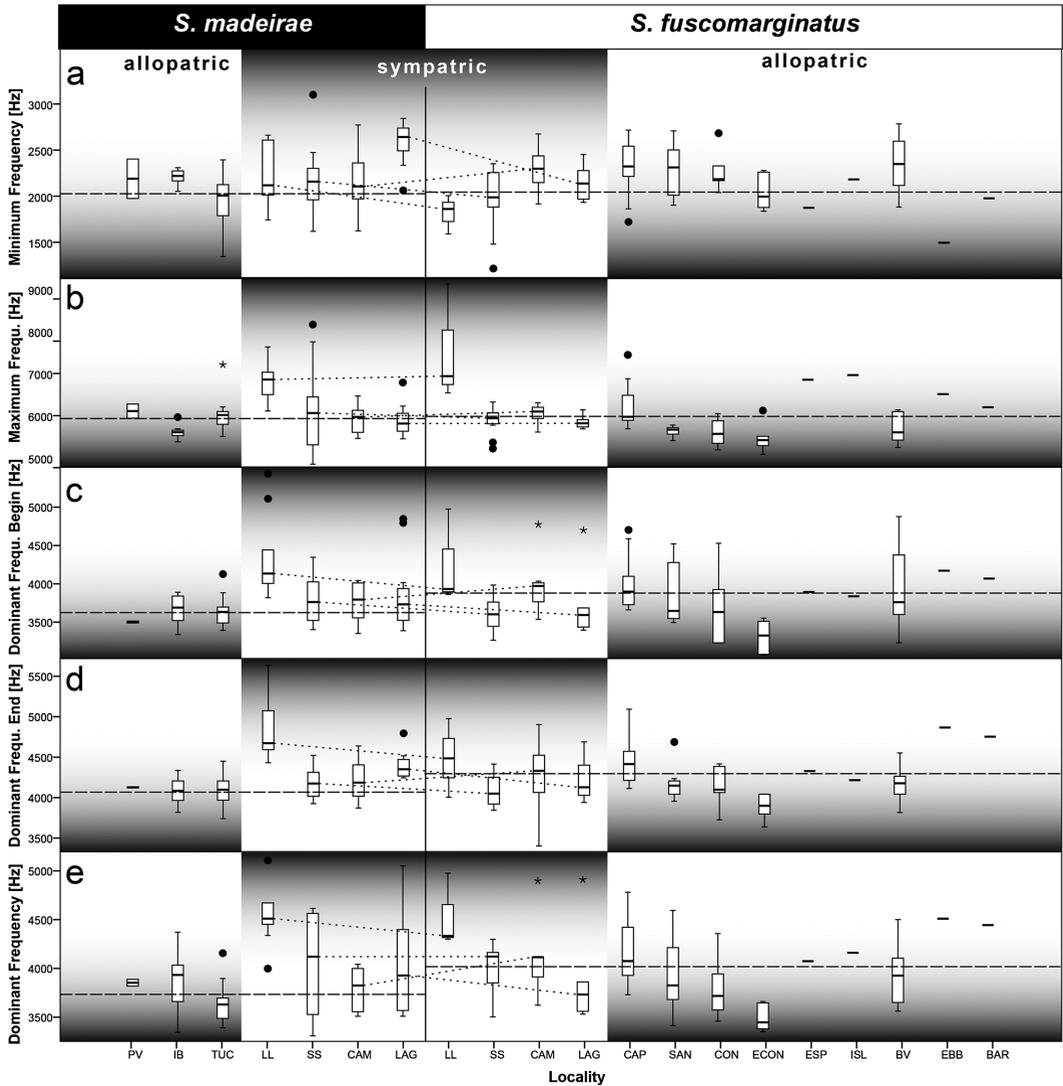


Figure 4. Original call traits (spectral parameters) of *Scinax madeirae* and *S. fuscomarginatus* at allopatric and sympatric locations. Dashed lines indicate means of allopatric locations. Calls of the two species diverge in sympatry in (a) MNF, (b) MXF, (c) DFB, (d) DFE, and (e) DF. Abbreviations of locations (in order of appearance; see as well fig. 2 for more details): PV: Porto Velho, Rondônia, Brazil; IB: Inselberg, Santa Cruz, Bolivia; TUC: Tucuman, Santa Cruz, Bolivia; LL: Los Lagos (Selvabue), Beni, Bolivia; SS: Centro de Investigaciones Ecológicas Chiquitos, San Sebastián, Santa Cruz, Bolivia; CAM: Campamento, Santa Cruz, Bolivia; LAG: Lagunitas, outpost of Hacienda Caparú, Santa Cruz, Bolivia; CAP: Hacienda Caparú, Santa Cruz, Bolivia; SAN: San Ignacio de Velasco, Santa Cruz; CON: Concepción, Santa Cruz; ECON: 9.4 km E Concepcion, Santa Cruz, Bolivia; ESP: El Espinal, Santa Cruz, Bolivia; ISL: Isla Bonita, near Santa Cruz de la Sierra, Santa Cruz, Bolivia; BV: Buenavista, Santa Cruz, Bolivia; EBB: Estación Biológica Beni, Beni, Bolivia; BAR: Barracón, Pando, Bolivia.

between sympatric and allopatric populations, with *S. madeirae* being generally larger than *S. fuscomarginatus*, but to a lesser extent in sympatry, while in *S. fuscomarginatus* SVL is more conserved (table 2).

Principal component analyses and MANOVAs

The PCA of original call data revealed three PCs with an eigenvalue > 1.0, explaining 76.8% of the total variance (PC1, eigenvalue = 3.00,

Table 3. Rotated component matrix showing character loadings and percentages of explained variance for Principal Components (PC) 1-3 for nine bioacoustic variables. The PC analysis was applied to z -scores of original data and their residuals, where effects of SVL and temperature had been statistically removed using a preparatory MANCOVA (see text for details).

	Original data			Residual data		
	PC 1	PC 2	PC 3	PC 1	PC 2	PC 3
MNF	0.109	0.004	0.926	0.125	0.035	-0.920
MXF	0.578	0.129	0.600	0.249	0.438	0.686
DF	0.929	-0.035	0.138	0.092	0.909	0.197
DFB	0.916	-0.091	-0.044	-0.036	0.935	0.014
DFE	0.896	-0.020	-0.003	0.162	0.852	0.104
PR	0.020	-0.957	0.056	-0.943	-0.107	0.074
PD	-0.007	0.898	0.080	0.879	0.095	0.051
CD	0.010	0.961	0.018	0.945	0.118	0.026
CR	0.157	-0.256	0.224	-0.201	0.131	0.175

33.3% variance explained; PC2, eigenvalue = 2.70, 30.0%; PC3, eigenvalue = 1.22, 13.5%). PC1 was highly loaded with spectral parameters, PC2 with temporal parameters, and PC3 with spectral parameters (table 3). Likewise, the PCA on corrected data (residuals) retrieved three PCs with an eigenvalue > 1.0, explaining 75.4% of the total variance (PC1, eigenvalue = 3.36, 37.4% variance explained; PC2, eigenvalue = 2.25, 25.0%; PC3, eigenvalue = 1.17, 13.0%; table 3). PC1 was highly loaded with temporal parameters, whereas spectral parameters had high loadings on PC2 and PC3 (table 3).

MANOVA using these three PCs as dependent variables uncovered a highly significant effect of the main factor “species” in both analyses (table 4). We used separate GLMMs on individual PCs to explore this effect further. The main factor “species” was highly significant for one PC in both analyses (table 5); this was PC2 in the analysis of original data, and PC1 in the analysis on residuals (see above; table 5). Characters with the highest axis loadings in these PCs were the temporal characters CD (in positive direction), PR (negative direction) and PD (positive direction; table 3). This effect is depicted in fig. 5, where *S. madeirae* and *S. fuscumarginatus* are clearly separated along PC1 in the analysis of residual data (not shown for raw data).

Table 4. MANOVA results for differentiation in (A) original call parameters and (B) size- and temperature-corrected residuals of call traits of *Scinax madeirae* and *S. fuscumarginatus*. “Sympatry”/allopatry and species identity (“species”) were treated as fixed effects. F -ratios were approximated using Wilks’ λ values.

Effect	Wilks’ λ	df	F	P
(A) Original data				
Species	0.166	3, 128	214.80	<0.0001
Sympatry	0.950	3, 128	2.24	0.087
Species \times sympatry	0.865	3, 128	6.66	<0.0001
(B) Corrected data				
Species	0.311	3, 128	94.41	<0.0001
Sympatry	0.958	3, 128	1.86	0.14
Species \times sympatry	0.861	3, 128	6.90	<0.0001

In the MANOVA the interaction term “species \times sympatry/allopatry” also was highly significant in both analyses (table 4). Post hoc GLMMs confirmed a significant interaction effect in the analysis of PC3 for both data-sets (table 4). Characters with the highest axis loadings in these PCs were the spectral parameters MNF (positive direction in both analyses) and MXF (positive in the analysis of the original data, negative in the analysis of residuals; table 3).

Hence, we found evidence for character displacement in sympatric compared to allopatric populations, but this effect differs between species. Visualization via PC plots shows a separation between sympatric and allopatric populations in *S. madeirae*, but not in *S. fus-*

Table 5. Results of generalized mixed models based on PC 1-3 for differentiation of call traits (see text for details) of *Scinax madeirae* and *S. fuscomarginatus*.

Source	Original data			Residual data		
	df	<i>F</i>	<i>P</i>	df	<i>F</i>	<i>P</i>
PC1						
Intercept	1, 10 331	0.003	0.96	1, 6884	0.94	0.36
Species	1, 18 603	0.11	0.75	1, 16 497	153.70	<0.0001
Sympatry	1, 10 331	2.16	0.17	1, 6884	0.31	0.60
Species × sympatry	1, 18 603	2.29	0.15	1, 16 497	0.26	0.62
PC2						
Intercept	1, 11 233	0.70	0.42	1, 6688	0.24	0.64
Species	1, 19 433	293.05	<0.0001	1, 19 170	0.14	0.71
Sympatry	1, 11 233	0.47	0.51	1, 6688	1.34	0.29
Species × sympatry	1, 19 433	0.72	0.41	1, 19 170	4.08	0.058
PC3						
Intercept	1, 7341	0.626	0.45	1, 8485	0.80	0.40
Species	1, 19 170	0.897	0.36	1, 20 722	0.59	0.45
Sympatry	1, 7341	0.012	0.91	1, 8485	0.09	0.77
Species × sympatry	1, 19 170	4.374	0.050	1, 20 722	4.78	0.040

comarginatus (fig. 5), suggesting that only *S. madeirae*, but not *S. fuscomarginatus* shows character displacement in sympatry. These results confirm our qualitative observations based on original call data (see above) and demonstrate that the dissimilarity of *S. madeirae* calls between sympatric and allopatric populations is achieved by displacement of several single parameters, but affects only spectral characters.

Variability of call characteristics

Our assessment of relative individual variability found that in both species intra-individual variances (CV_{wi}) of MNF and CR were highest, while the temporal parameter PR had the lowest CV_{wi} (fig. 6). The low CV_{wi} in PR renders this parameter a prime candidate for individual recognition, even though our study provides no data or experiments to support or reject this hypothetical function.

Empirical tests of different hypotheses seeking to explain why only one of the two species investigated here shows character displacement (i.e., *S. madeirae*) remained inconclusive; results are presented in the online Supplements 1-3.

Discussion

We found evidence for character displacement in the acoustic mating signal of *Scinax madeirae* in situations of sympatry with the closely related *S. fuscomarginatus*. The present study uncovered the following pattern: both species differ in temporal parameters in allopatry, as well as in sympatry, and thus, these variables did not change in the sympatric situation; however, we found a shift in spectral call parameters in sympatry that are rather similar in allopatry. Moreover, the identified character displacement showed an interesting pattern where spectral parameters of *S. madeirae* are lower than those of *S. fuscomarginatus* in allopatry, but higher in the sympatric situation.

Why are only spectral, but not temporal characters displaced? A plausible explanation is that there is little divergent selection on temporal traits as species differences are already pronounced in the allopatric situation. Temporal traits, especially pulse rate, are often said to be responsible for species recognition (e.g. Gerhardt and Huber, 2002), and species identification through temporal parameters is possibly already guaranteed so that the risk of misidentification based on temporal parameters is already

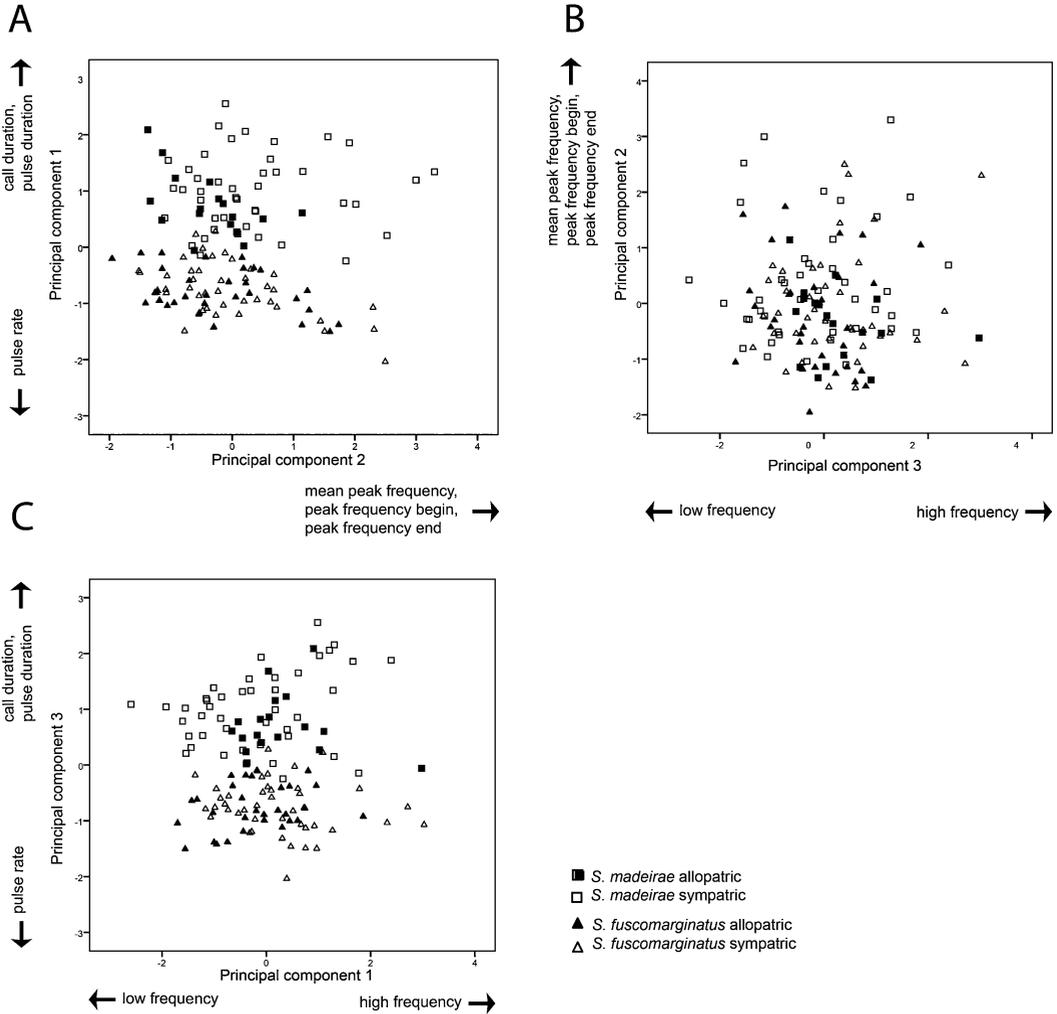


Figure 5. Principal component analysis (PCA) plot based on nine temperature- and size-corrected residuals of call variables of *Scinax madeirae* (full square: allopatric; open square: sympatric) and *S. fuscomarginatus* (full triangle: allopatric; open triangle: sympatric) in allopatry and sympatry. Highest axis loadings are given beneath the axes.

rather low. An additional, not mutually exclusive, explanation for why only spectral characters diverged in sympatry comes from our analysis of individual variation of call traits, which suggests that at least some temporal traits could have a function in individual recognition. PR in particular was far less variable than other traits within *S. madeirae* individuals, which renders this trait a prime candidate for individual recognition (“individual signature”; Gerhardt, 1991; Bee et al., 2001; Feng et al., 2009; Gasser, Amézquita and Hödl, 2009). Thus, stabilizing

selection on these recognition cues due to functions other than mate attraction could constrain them for being displaced in sympatry.

Theoretically, the observed shift in spectral traits towards higher frequencies could be a by-product of ecological selection on body size – given that sympatric *S. madeirae* have a smaller body size than allopatric populations (table 2) – and thus, could be an incidental consequence of interspecific competition (alluding to a more important role of ECD). Alternatively, the shift in spectral traits could be a specific evolution-

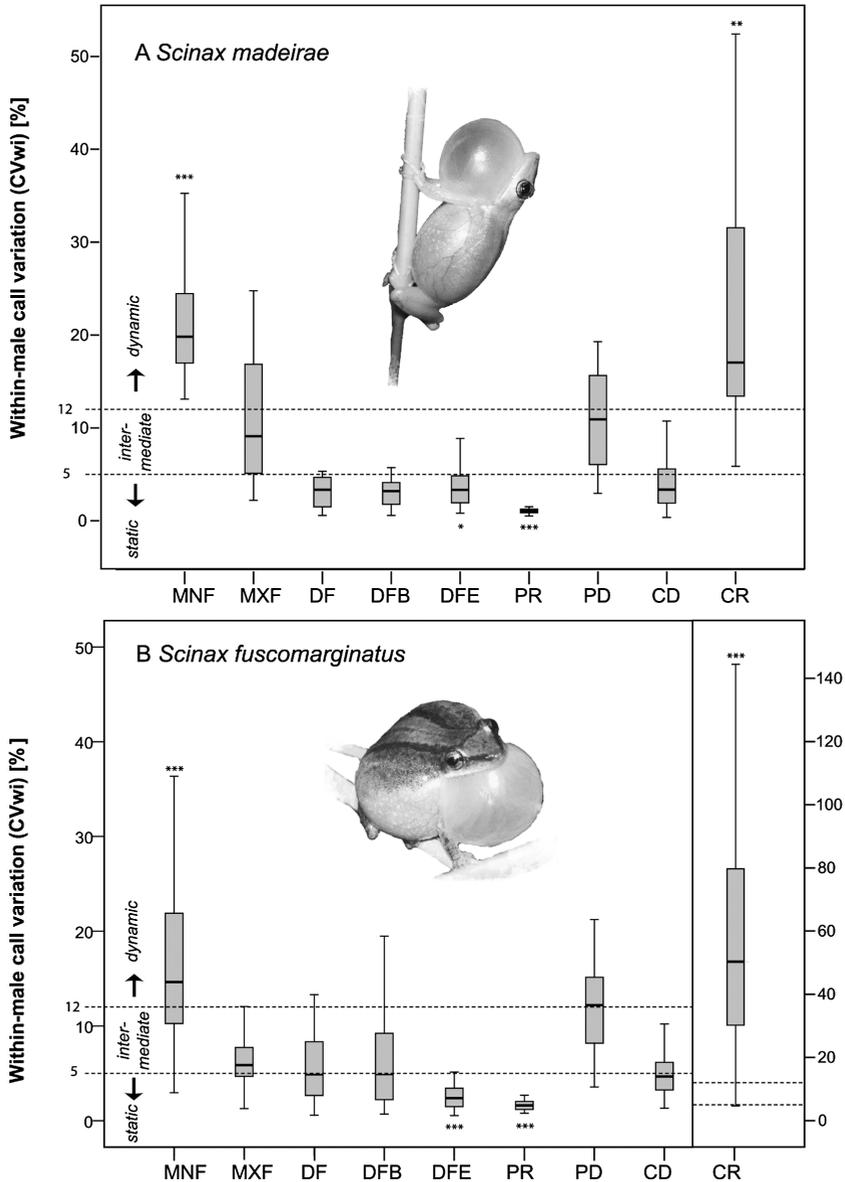


Figure 6. Within-male call variation (CV_{wi}) in (A) *Scinax madeirae* (16 individuals; 102 calls) and (B) *S. fuscomarginatus* (41 individuals; 313 calls). Boxplots indicate the interquartile range of the sample (height of box), the median (black line in box), and sample maximum and minimum (crossbar at the end of whiskers; outliers and extreme values not shown).

ary response to selection in order to reduce heterospecific sexual interactions (RCD). However, even if RCD was the driving force behind the observed shift in spectral traits, selection could still affect call characters indirectly, and the direct target could be (smaller) body size with consequently more divergent calls (higher frequency). We argue against those hypotheses,

as our analyses suggest that body size alone is not responsible for the observed frequency shift in sympatric *S. madeirae*. Even when we eliminated size and temperature effects and used residual data for statistical analysis, we still found an effect of character displacement in sympatry (see interaction effect of “species × sympatry/allopatry” in the MANOVA; table 4).

Another hypothesis that we tested in our present study is that selection for RCD targets larval size independent of body size (McClelland, Wilczynski and Ryan, 1998). Our results do not support this idea (online Supplement 6), even though sample sizes were small, and so the results have to be interpreted with caution. Hence, we tentatively argue that the shift in frequency-related traits represents a behavioral response. A variety of taxa have been shown to avoid interference or masking in noisy environments by vocal compensation (primates: e.g. Brumm et al., 2004; fish: e.g. Slabbekoorn et al., 2010; cetaceans: Lesage et al., 1999; Miller et al., 2000; Foote, Osborne and Hoelzel, 2004; Parks, Clark and Tyack, 2007; Parks et al., 2011; frogs: Sun and Narins, 2005; Lengagne, 2008; Parris et al., 2009; Cunningham and Fahrig, 2010). In many cases (primates, birds), signal masking effects were counteracted by increasing sound level (the so called “Lombard effect”, Lombard, 1911), possibly to maintain a specific signal-to-noise ratio that is favourable for signaling or a given space for communication (Brumm, 2004; Brumm et al., 2004; for reviews see Brumm and Slabbekoorn, 2005; Brumm and Zollinger, 2011). Although evidence for the Lombard effect in frogs is still lacking (Love and Bee, 2010; Brumm and Zollinger, 2011), however, plastic vocalization responses to background noise by increasing call rate or frequencies exist in various frogs (Langagne, 2008; Cunningham and Fahrig, 2010). As well, heterospecific sounds may represent sources of masking interference, and acoustic niche partitioning, e.g. spectral-partitioning, in complex assemblages can be observed (e.g. Hödl, 1977; Duellmann and Pyles, 1983; Amézquita et al., 2011). A recent study indicates that some frogs facultatively vary call frequency to avoid overlap with the spectral components of syntopic species (Both and Grant, 2012). This hypothesis, as well as the possibility that there is a heritable component to this behavioral variation, clearly warrants future experimentation by investigating context-dependent vocalization in *S.*

madeirae and examining vocalization of common garden-reared frogs. Additionally, future experiments will need to examine if the small shift in frequency mitigates acoustic interference between the two species at all. Moreover, the courtship signal may be multimodal (e.g., acoustic and visual: Rosenthal, Rand and Ryan, 2004; Taylor, Buchanan and Doherty, 2007) and composite, including visual cues (e.g. vocal sac movement), olfactory or dynamic (tactile) components (Partan and Marler, 2005), but also different microhabitat choice (like different perch heights) could play a role. Those possible additional components of the male courtship signal, however, were not the focus of this study. Similarly, there is a potential effect of call intensity that was not measured in this study. Possibly, the species with the lowest source level would be the one that showed displacement. Moreover, shifts in female preferences in sympatry may be responsible for the displacement found in this study (e.g. Marquez and Bosch, 1997).

An unusual finding in the present study is the pattern of frequency displacement in *Scinax madeirae* with relative lower frequencies in allopatry, but higher frequencies in sympatry. We argue that the simultaneous action of ecological competition and RCD could explain this rare pattern. *Scinax madeirae* becomes smaller in sympatry with *S. fuscomarginatus*, possibly due to an effect of interspecific resource competition. However, as a result of smaller body size, these populations cannot show the spectral calling behavior they show in allopatry (in which case they would basically interfere with the similar frequencies of *S. fuscomarginatus*). On the other hand, producing even lower frequencies may be constrained by the small body size of sympatric *S. madeirae*, resulting in a shift towards higher frequencies. In addition to selection to avoid hybridization, the observed character displacement could be driven by short-term behavioral plasticity to prevent interference for the same reason (avoidance of hybridization).

We are aware of a number of alternative explanations, and so future studies will need to

consider sender and/or receiver morphology and neurophysiology, community composition and the acoustic environment (e.g. community-wide character displacement or overdispersion; e.g., Dayan and Simberloff, 2005), and also physical habitat features, as well as the phylogenetic history of a given population (reviewed in Wilkins, Seddon and Safran, 2013).

Our finding of character displacement in mating signals in sympatric *Scinax* frogs implies some form of selection to avoid hybridization, e.g., due to reduced hybrid viability (Dobzhansky, 1937, 1940; Howard, 1993; Coyne and Orr, 2004), and so future experiments will be needed to explore the extent of genetic incompatibility in this system. Reduced hybrid fitness (i.e., postzygotic isolation) ought to be strengthened by RCD (i.e., prezygotic isolation), and indeed, we found only one – if any – possible hybrid in a rather large sample that was assigned to *S. madeirae* during a BLAST search (based on maternally inherited mtDNA), but had intermediate call characteristics (Supplement 4). This finding shows, on the one hand, that hybridization is still possible in this mating system despite mechanisms to reduce hybridization, but is a rare event; on the other hand, an intermediate hybrid call (which might not attract either type of female) demonstrates how costly hybridization in that mating system is.

Overall, our study system is particularly suitable to investigate the factors limiting and determining the direction of signal evolution in secondary contact zones. Our study suggests the potential role of individual recognition in determining and constraining the traits under divergent selection in secondary contact zones, but highlights the need to determine the factors responsible for the observed asymmetry in RCD on the species level (i.e., why does only one species show RCD?). Future studies will greatly profit from integrating phylogeographic information, female phonotaxis experiments, male individual-recognition experiments, common garden-rearing of different male types, investigations of niche segregation, and in depth

investigations of the acoustic space in this study system.

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Online supplementary material

We made an attempt to answer the question of why displacement does not affect both species equally (asymmetric vs. symmetric displacement; Schluter, 2000; Cooley, 2007), and if one can predict the displacing species. We conducted empirical tests of different hypotheses seeking to explain the pattern of only one of the two species (i.e., *S. madeirae*) showing RCD. Results from two approaches to answer this question remained inconclusive and are presented in Supplements 1 and 2; additional hypotheses that will need to be addressed in future studies are listed in Supplement 3.

Results from BLAST searches to verify the species identity of one individual that showed

intermediate call characters (table S4.2) are presented in Supplement 4.

Additional results from our analysis of different call parameters are presented in Supplement 5. While the main manuscript shows only spectral parameters in allo- and sympatric *S. madeirae* and *S. fuscomarginatus* populations, the supplementary fig. S5.1 depicts temporal call parameters.

Finally, Supplement 6 provides the results from a morphological analysis of larynx size to test the hypothesis that larynx size variation independent of body size variation may explain (all or parts of) the displacement of spectral characters in *S. madeirae*.

A list of the recordings and their according data, as well sound archive reference numbers from the Tierstimmenarchiv, Berlin, and the according links to the sound files can be found in the Supplement 7.

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