

Asymmetric frequency shift in advertisement calls of sympatric frogs

Martin Jansen^{1,2,*}, Martin Plath³, Francisco Brusquetti^{4,5}, Michael Joseph Ryan²

¹Section of Herpetology, Senckenberg Gesellschaft für Naturforschung, Senckenberganlage 25, 60325 Frankfurt am Main, Germany

²Section of Integrative Biology, University Station C0930, University of Texas, Austin TX 78712, USA

³Northwest A & F University, Department of Animal Science, Shaanxi, China

⁴Departamento de Zoologia, Instituto de Biociências, Universidade Estadual Paulista, UNESP, Cx. Postal 199, 13506-906 Rio Claro, São Paulo, Brasil

⁵Instituto de Investigación Biológica del Paraguay, CP 1429, Asunción, Paraguay

Supplementary Material

Supplement 1. Estimation of population densities in sympatric populations

Introduction

Empirical studies suggest that an asymmetry of divergence in secondary contact zones is typical for RCD, with only one species (generally the same species across sites) showing displacement (Cooley, 2007; Jang, 2008). Asymmetry in RCD thus is a common phenomenon, and potential explanations for this asymmetry are manifold. For example, differences in species abundance—whether recent or at the time of first contact—may affect the evolution of RCD, as the rarer species faces a disproportionately higher risk of mismatched mating (e.g., Littlejohn, 1965; Goldberg and Lande, 2006; Kirschel, et al. 2009). We therefore examined relative abundances of both species at localities where they occur in sympatry and asked if the less abundant species would be more likely to diverge (see Cooley, 2007 and Jang, 2008 for a review).

Material and methods

At three localities with sympatric occurrences of both species [San Sebastián (site 10 in fig. 1), Caparú (15), and Campamento (16)] we could estimate population densities of both species by determining densities of calling males using Audio Strip Transects (AST) following Zimmerman (1994). Fourteen to 34 transects of 25 m length were set up at each locality. Transect width was three meters on both sides of the AST. The surveys were conducted by walking slowly in one direction along a given transect while counting all calling males within the pre-defined counting strip. To compare densities of both species in three sympatric populations obtained from our transect counts we used paired *t*-tests.

Results and discussion

While *S. fuscomarginatus* was significantly more abundant than *S. madeirae* in Caparú, there was no significant difference in Campamento, and in San Sebastián *S. madeirae* was significantly more abundant than *S. fuscomarginatus* (fig. S1.1). Hence, we did not find support for the hypothesis that the rarer species is displacing. However, asymmetric displacement could be a ‘fossil’ of past selection (Grant, 1972; Schluter, 2000; Cooley, 2007), e.g., if *S. madeirae* invaded later in the contact zone. Moreover, frog populations are known to be fluctuating, and the densities found during our study may as well not reflect long term means.

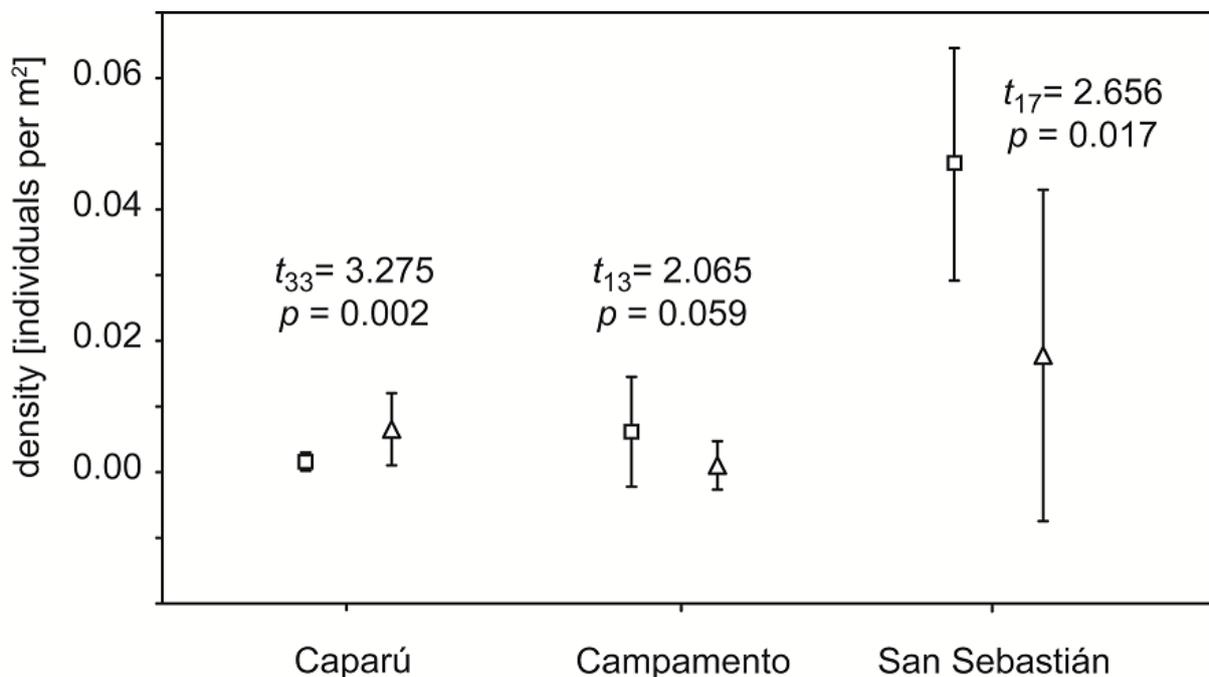


Figure S1.1. Mean (\pm SD) densities of *Scinax madeirae* (open square) and *S. fuscomarginatus* (open triangle). Densities were compared using *t*-tests.

Supplement 2. Analyses of variability of call traits in allopatric populations

Introduction

Differential responses between species to the co-occurrence of the respective other species (namely, a signal of character displacement in *S. madeirae* but not *S. fuscomarginatus*) could be explained through higher standing variation (and thus, evolvability) in allopatric *S. madeirae* populations. We therefore 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).

Material and methods

We tested the null hypothesis of homogeneity of variances of spectral call parameters (those that are displaced in *S. cf fuscomarginatus*) in allopatric populations using Levene's tests. We used residuals that were corrected for potential temperature and size effects (see main text).

Results and discussion

We found variances of most variables to not differ between species, the sole exception being the variance in dominant frequency at the beginning of a call (DFB); however, variance in this call characteristic was higher in *S. fuscomarginatus* (variance = 0.89), not *S. madeirae* (variance = 0.33; Table S2.1).

Table S2.1. Levene's Test for homogeneity of variances in spectral parameters of allopatric populations of the two studied frog species.

Residuals of parameters	Variance		Test of homogeneity of variances		
	<i>S. madeirae</i>	<i>S. fuscomarginatus</i>	Levene statistic	df	<i>P</i>
MNF	0.243	0.348	1.04	1, 62	0.31
MXF	0.173	0.141	0.02	1, 62	0.89
DF	0.413	0.594	1.06	1, 62	0.31
DFB	0.328	0.892	5.09	1, 62	0.028
DFE	0.257	0.744	3.35	1, 62	0.072

Hence, we could not find evidence for the hypothesis that the displacing *S. madeirae* has more variation in the displaced characters in allopatry (which could facilitate evolution of these characters, assuming that high character variation reflects high genetic variability, see

Slatkin, 1980; Pfennig and Pfennig, 2009). We used call variation as a proxy for genetic variation, but are aware that further studies are required to test actual genetic variation (at loci putatively under divergent selection in sympatry) as well as other potential factors influencing this variation (e.g., hormone levels, ecological factors).

Supplement 3. Additional hypotheses for asymmetrical RCD

Further explanations for the asymmetrical pattern could be differences in the timing of range expansions into the contact zone, probably related to abundance bias at the beginning of establishment of the species: Otte (1989) and Littlejohn (1999) suggested that the species that expanded its range into that of the other ought to show character displacement. Also differences regarding the costs of hybridization (Lemmon, 2009), homogenization of divergence by gene flow (i.e. spreading of divergent characters of sympatric *S.*

fuscocomarginatus into allopatric populations and vice versa, obscuring potential character displacement in this species; Jang and Gerhardt, 2006), and different morphological or phylogenetic constraints to signal evolution have been invoked to affect asymmetrical RCD (see Wilkins, et al. 2013).

Supplement 4. Detection of a hybrid individual

For some of the specimens included in this study ($N = 35$ out of a total $N = 134$ individuals) species identity was already verified using a barcoding approach (see data in Jansen, et al. 2011). We complemented this data set with a newly generated sequence (GenBank accession KT334161), because this individual showed remarkable call characteristics: call duration was similar to *S. fuscocomarginatus*, and pulse rate was more similar to *S. madeirae* (table S4.1; please compare with call characteristics of *S. madeirae* and *S. fuscocomarginatus*; table 1 in the main text). Moreover, the call had a somehow harsh, abnormal and inordinate sound (e.g., pulse rate was highly variable). We hypothesized that this individual might be a hybrid, or it could be an undescribed divergent lineage.

DNA was extracted from muscle tissue using a glass fiber extraction protocol (Ivanova, et al. 2006). We used the primers 16SA (forward): 5'-CGCCTGTTTATCAAAAACAT, and 16SB-H (reverse): 5'-CCGGTCTGAACTCAGATCACGT; Vences et al. 2005) to amplify a fragment of the mitochondrial 16S rRNA gene (550–600 bp) using a Mastercycler® pro S (Eppendorf) under cycling conditions described elsewhere (Jansen, et al. 2011). We conducted a search of sequences deposited in the GenBank DNA database by using the BLAST algorithm (<http://www.ncbi.nlm.nih.gov/BLAST/>; Altschul, et al. 1997) in order to obtain an approximation of species identity.

BLAST search indicated genetic (mt16S) assignment of sample MNKA 9950 to *Scinax fuscomarginatus* (table. S4.1), rendering the possibility unlikely that this specimen belongs to an as yet unknown divergent lineage. Future studies with other markers will need to rigorously test the hypothesis that this individual is indeed a hybrid.

Table S4.1. Descriptive statistics [mean \pm SD (min–max)] of eleven calls of individual MNKA 9950. Shown are original data, not corrected for SVL and ambient temperature.

Call trait	mean \pm SD (min - max)
MNF (Hz)	2265 \pm 240 (2002 – 2696)
MXF (Hz)	6422 \pm 494 (5724 – 7479)
DF (Hz)	3633 \pm 112 (3445 – 3842)
DFB (Hz)	3732 \pm 347 (3445 – 4694)
DFE (Hz)	4252 \pm 28 (4221 – 4307)
PR (Hz)	141 \pm 26 (113 – 200)
PD (ms)	3.8 \pm 1.1 (2.6 - 6.8)
CD (ms)	265 \pm 13 (243 – 291)
CR (Hz)	0.99 \pm 0.88 (0.01 - 1.65)
SVL (mm)	22.8

Table S4.2. First 10 BLAST search hits for sequence of individual MNKA 9950 from Las Lagunitas, Bolivia (see fig. 2; KT334161). Please note: Species names are as cited in Jansen et al. (2011), *Scinax parkeri* corresponds to *S. fuscomarginatus* following Brusquetti et al. (2014).

Description	Maximum BLAST score	Identity	Accession
<i>Scinax parkeri</i> isolate MJ1363 16S ribosomal RNA gene, partial sequence; mitochondrial	1026	98 %	JF790010.1
<i>Scinax parkeri</i> isolate AS0264 16S ribosomal RNA gene, partial sequence; mitochondrial	1024	98 %	JF789984.1
<i>Scinax parkeri</i> isolate AS0313 16S ribosomal RNA gene, partial sequence; mitochondrial	1024	98 %	JF789987.1
<i>Scinax parkeri</i> isolate AS0315 16S ribosomal RNA gene, partial sequence; mitochondrial	1024	98 %	JF789989.1
<i>Scinax parkeri</i> isolate MJ1362 16S ribosomal RNA gene, partial sequence; mitochondrial	1022	98 %	JF790009.1
<i>Scinax parkeri</i> isolate MJ1251 16S ribosomal RNA gene, partial sequence; mitochondrial	1022	98 %	JF790000.1
<i>Scinax parkeri</i> isolate AS0325 16S ribosomal RNA gene, partial sequence; mitochondrial	1022	98 %	JF789990.1
<i>Scinax parkeri</i> isolate AS0265 16S ribosomal RNA gene, partial sequence; mitochondrial	1022	98 %	JF789985.1
<i>Scinax parkeri</i> isolate MJ887 16S ribosomal RNA gene, partial sequence; mitochondrial	1022	98 %	JF789994.1
<i>Scinax parkeri</i> isolate AS0326 16S ribosomal RNA gene, partial sequence; mitochondrial	1022	98 %	JF789991.1

Supplement 5. Additional results from analysis of different call parameters

Temporal call parameters in allo- and sympatric *Scinax madeirae* and *S. fuscomarginatus* populations are shown in fig. S5.1.

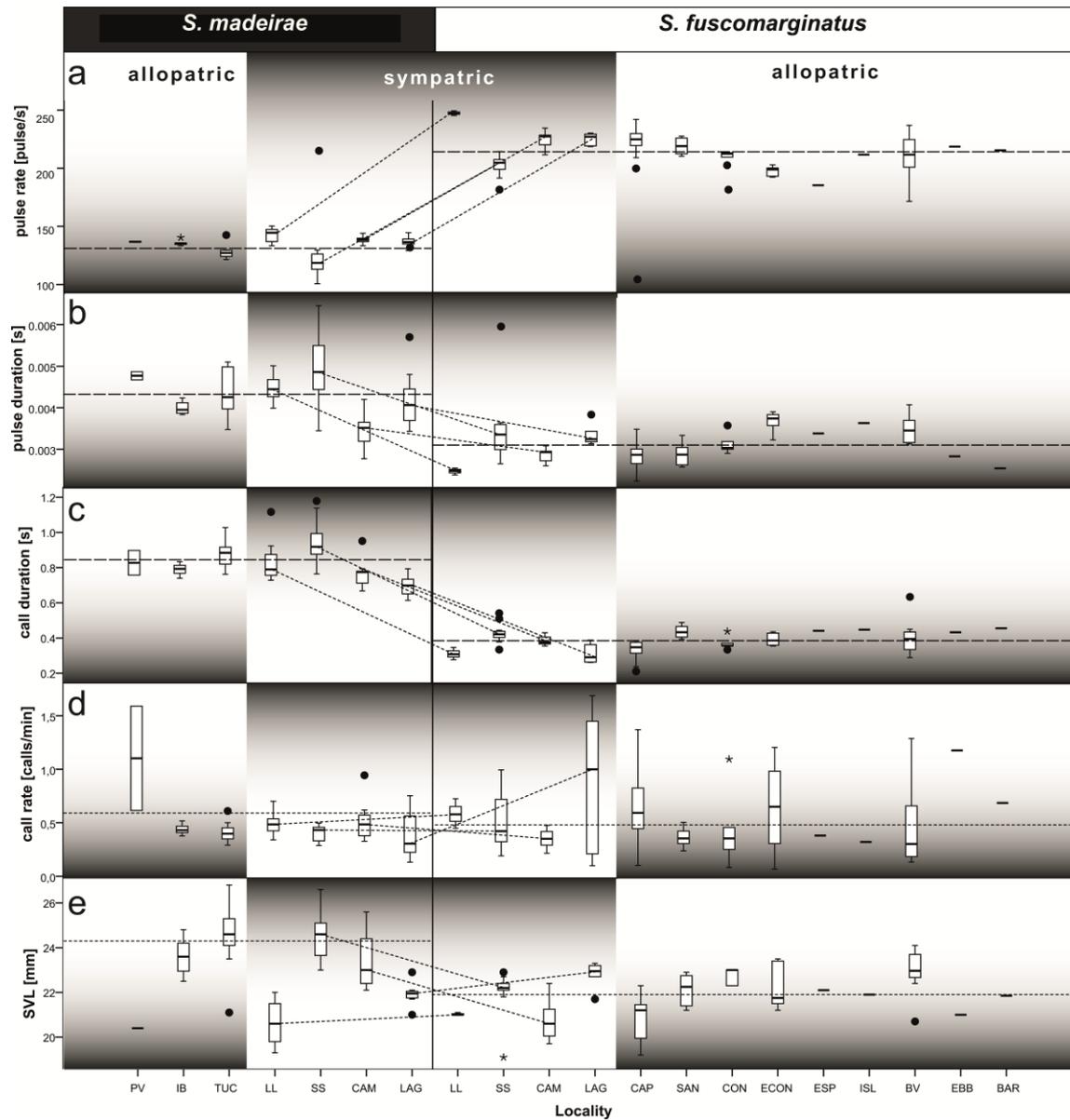


Figure S5.1. Original temporal call parameters and body size of *Scinax madeirae* and *S. fuscomarginatus* in allopatric and sympatric situations. Dashed lines indicate means of allopatric locations.

Supplement 6. Is there specific selection on larynx size in sympatric *Scinax madeirae*?

Introduction

We identify displacement only in spectral parameters which are known to be correlated with body size in frogs (Zweifel, 1968), and indeed, we found slight body size differences between allopatric and sympatric *S. madeirae* populations. However, our analysis of free-size call traits also identifies differences between allopatric and sympatric *S. madeirae* populations, which led to the question of whether larynx size evolves independent of body size. On the other hand, given that the larynx is only one part of a well-integrated, complex sound-producing organ (Kime, et al. 2013), increasing body size and thus all the components of the vocal production system might be easier to accomplish than increasing larynx size alone.

Thus we determined if body-size differences alone can account for the frequency shift in that species, or if larynx size variation independent of body size variation may explain (all or parts of) the displacement of frequency-related characters in *S. madeirae*.

Material and methods

We dissected 16 specimens of *S. madeirae*, six from two allopatric sites and 10 from three sympatric sites for a preliminary examination of laryngeal anatomy. We excised the hypolaryngeal apparatus and removed all connective tissue and muscles surrounding the larynx to expose the cricoid cartilage, arytenoid cartilage, and bronchial passages (McClelland et al. 1998; Boul and Ryan 2004). As a proxy for larynx size we measured lateral larynx length under a Leica Stereomicroscope M205 C and using the software Leica Application Suite (see fig. S6.1).

In a first step we used an ANOVA to test if there is a difference in larynx size (lateral length) between allopatry and sympatry. In a second step we tested if this difference still exists once body size is controlled for. For this purpose we computed each a regression between larynx size and body size (snout-vent length, SVL) in allopatry and sympatry. We then tested for differences in intercepts (factor 'sympatry'/allopatry) and potential slope heterogeneity (interaction term) with a General Linear Model (GLM) that included 'body size' as a covariate. We then ran a reduced model, while excluding the non-significant ($F_{1,16} = 0.38$, $P = 0.55$) interaction term. All statistical analyses were conducted using SPSS 17.0.

Results and discussion

ANOVA detected no difference in larynx size between allopatric (3.99 ± 0.32 mm, $N = 6$) and sympatric populations (3.71 ± 0.34 mm, $N = 10$; $F_{1,16} = 2.83$, $P = 0.12$) when body size differences were not considered. In both cases, larynx size increased with increasing body size (linear regressions, allopatry: $R^2 = 0.64$; sympatry: $R^2 = 0.66$; fig. S6.2). GLM did not reject the assumption of slope homogeneity ($F_{1,16} = 0.38$, $P = 0.55$). The reduced model (interaction term removed) identify larynx size to not differ between allopatry and sympatry ($F_{1,16} = 0.65$, $P = 0.44$; estimated marginal means for allopatry: 3.87 ± 0.09 mm; sympatry: 3.78 ± 0.07 mm), while the covariate 'body size' had a significant effect ($F_{1,16} = 23.55$, $P < 0.001$).

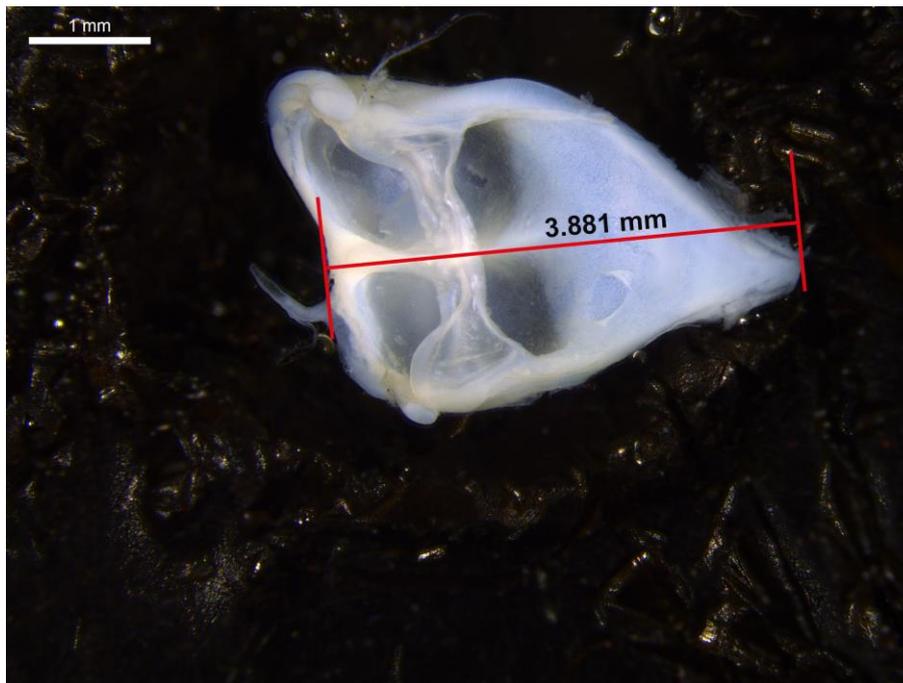


Figure S6.1. Lateral view of larynx of *Scinax madeira* lateral cut, specimen MJ-1109, with measurement of lateral larynx length. Scale = 1mm.

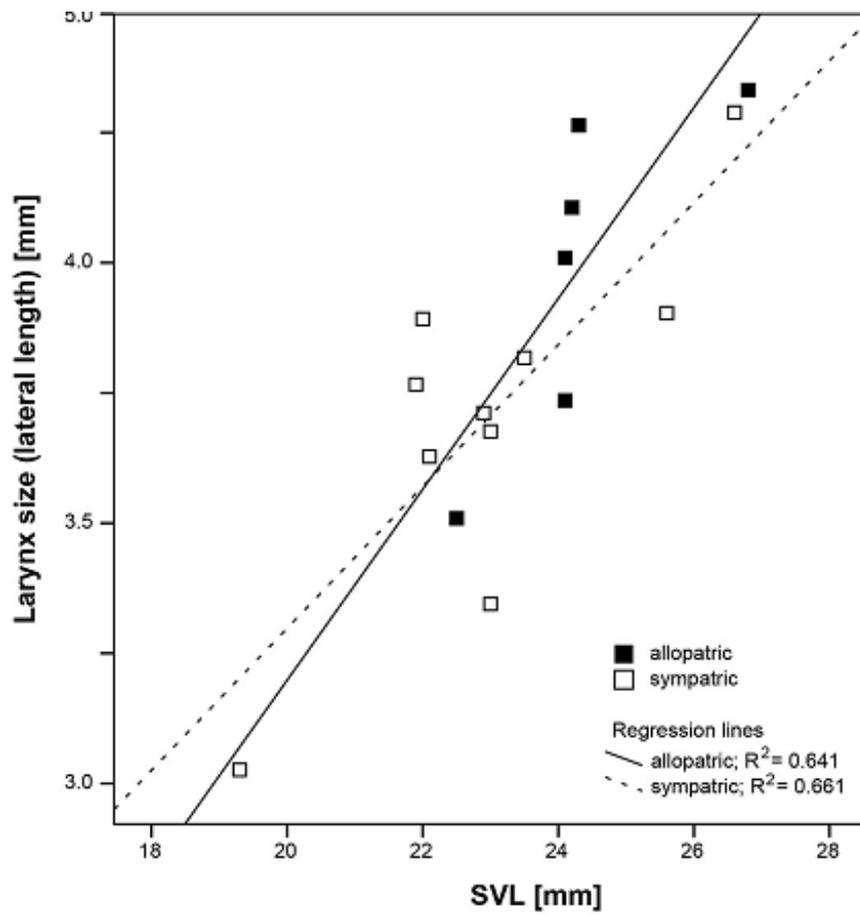


Figure S6.2. Linear regression lines of larynx size plotted against body size (SVL) in allopatric and sympatric *S. madeirae*.

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