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Beyond the river: underlying determinants of population acoustic signal variability in Amazonian direct-developing *Allobates* (Anura: Dendrobatoidea)

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Abstract The multidimensional nature of animal signals makes acoustic traits potentially subject to different determinants. The Amazonian frogs *Allobates nidicola* and *Allobates masniger* have an allopatric distribution, occurring along the left and right sides of the Madeira River, respectively. These are two sister, phenotypically similar species whose eggs are deposited and develop entirely in a terrestrial nest. In this study, we analyzed 2,000 advertisement calls recorded from ten localities across Central Amazonia, in order to understand the role of determinants of acoustic signal variability at the population and species levels. We assessed, through nested analyses of variance, the differentiation of six characters of this sexual signal among populations and between interfluves. Moreover, we measured the degree of variability and the extent of temperature- and body size-induced plasticity in call traits. We also tested for isolation-by-distance effects in phenotypic differentiation through Mantel tests. Coefficients of variation were higher among than within populations for all call measurements. Spectral call properties were more distinctive than temporal traits among populations and species. Advertisement call traits showed strong temperature-induced plasticity (e.g., 45 % of the variation in note duration). In contrast, the effects of body size were restricted to frequency-related characters. The river barrier effect was significant among all the acoustic variables analyzed even after controlling for male body size. Geography (sampling locality) and body size also jointly affected call variability. No correlation between geographical and acoustic distances among populations was observed, suggesting that local stabilizing selective pressures have an important role in the evolution of call differentiation.

Keywords Advertisement call · Geographical variation · Isolation by distance · River barrier effect · Sexual signal · Signal plasticity

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Introduction

Each of the properties of an acoustic signal typically shows a characteristic range of variation at different levels of analysis (e.g., within populations, among populations, and between species) (Castellano et al. 2002). Moreover, it is expected that the multidimensional nature of animal signals will make acoustic traits potentially subject to different determinants (Gerhardt 1991; Gerhardt and Huber 2002). By decomposing acoustic signals into their properties, we might be able to assess the agents responsible for call variation and to account for their effects depending on the question asked in a particular study system. Additionally, by measuring the degree of variability of quantitatively variable traits of frog calls in different levels, we can evaluate their potential for sexual selection and social recognition, and also understand the roles of particular acoustic characters in microevolutionary processes (e.g., Friedl and Klump 2002; Gasser et al. 2009; Bee et al. 2010).

Call traits show temperature- and body size-induced plasticity (Prestwich 1994). Environmental temperature is often

regarded as a cofactor in acoustic studies, and it is necessary to account for its effect, especially for ectotherms, in order to obtain more accurate measures of call differentiation when comparing individuals, populations, or species. The extent to which temperature affects call traits varies considerably in anurans, and tropical species, which undergo more subtle temperature variations, are less studied than are species in temperate regions (see Gerhardt and Huber 2002; Wells 2007). Another cofactor often controlled in comparative acoustic studies is male body size, which is known to affect predominantly frequency-related characters of the call (Ryan 1988).

Geography plays an important role in the evolution and differentiation of acoustic signals (Wycherley et al. 2002), but its effect may not be equivalent for all the properties of the call. It has been suggested that spatial variation takes place according to the degree of variability of a given acoustic character, with static properties exhibiting a more pronounced variation among populations (Castellano et al. 2000). Moreover, relationships between geography and covariables such as body size might produce interactive effects between determinants of call differentiation (Pröhl et al. 2007; Kaefer and Lima 2012). Biogeographical studies in Amazonia are revealing the effects of river barriers on call divergence for different frog species, both in overall acoustic variability (Simões et al. 2008; Tsuji-Nishikido et al. 2012) and in specific properties of the call (Kaefer and Lima 2012). However, isolation by distance is also recognized as an important mechanism in phenotypic divergence in Amazonian frogs (e.g., Lougheed et al. 2006; Pröhl et al. 2006; Amézquita et al. 2009). Because isolation-by-distance effects are inferred through correlations between phenotypic and distance measures, they can be masked by the presence of river barriers, which might lead to acoustic distances that are disproportionally greater than those produced by linear distance. Therefore, testing for the occurrence of isolation-by-distance effects might provide clues to inform the selection/drift debate regarding the evolutionary forces driving differentiation.

In an earlier study, we demonstrated the usefulness of advertisement calls in discriminating between the similar Amazonian species *Allobates nidicola* Caldwell and Lima 2003 and *Allobates masniger* Morales 2002 (Tsuji-Nishikido et al. 2012). This study indicated that the lower portion of the Madeira River is an important biogeographical barrier involved in phenotypic differentiation, and suggested that *A. nidicola* and *A. masniger* have an allopatric distribution, occurring along the left and right sides of the river, respectively. Molecular genetic data confirm this proposed distribution and provide evidence that they constitute sister species (Kaefer et al. unpublished data). *A. nidicola* and *A. masniger* are two diurnal, ground-dwelling species whose eggs are deposited and develop entirely in a

terrestrial nest, and their adults share all the available morphological diagnostic characters (Morales 2002; Caldwell and Lima 2003) (Fig. 1). Tsuji-Nishikido et al. (2012), as in most taxonomic studies, limited themselves to summarizing the call measurements by applying principal components analyses, thus overlooking the individual contribution of each call trait to the differentiation between populations or species. Moreover, possible effects of isolation by distance were not addressed. Therefore, in this study we characterized the underlying determinants of population acoustic variability, by exploring this study system through the following questions: (1) What is the degree of variability of each of the call traits among populations and between species? (2) To what extent do temperature and body size induce call trait variability? (3) Is the river barrier effect significant among all the different acoustic variables? and (4) Does isolation by distance play a significant role in call divergence, beyond the known effects of the river as a barrier?

Material and methods

We analyzed a total of 2,000 calls from 100 males (20 calls per male) recorded at ten sites located in the Brazilian states


Fig. 1 *A. nidicola* (*top*) and *A. masniger* (*bottom*) males vocalizing at their respective type localities in Brazilian Amazonia

of Amazonas and Pará, including the type localities of *A. nidicola* and *A. masniger* (Fig. 2). Ten individuals were sampled in each locality, which were regarded as populations in this study. Data were collected between December 2009 and March 2010, during the rainy season, which encompasses the breeding periods of both species. Recordings were made using a Marantz PMD660 digital recorder (44.1 kHz sampling rate; 16-bit resolution) and an AKG 568EB directional microphone positioned 1–1.5 m in front of the calling male. After each recording, we measured environmental (air) temperature with a digital thermometer and captured the frog to measure body size (snout-vent length, SVL, in millimeters) with a digital caliper. The advertisement call of both species is composed of a single note repeated rhythmically over time, thus forming sequences of notes lasting from 2 to 5 min. A comparative graphical representation of the advertisement call of both species can be found in Tsuji-Nishikido et al. (2012). Call measurements were taken on Raven 1.2 (Window: Blackman; Discrete Fourier Transform: 1,024 samples; 3 dB filter bandwidth: 80.0 Hz) (Charif et al. 2004). The measurement used to represent each individual in the statistical analysis was the mean value of the parameters obtained from 20 notes and their respective intervals. We considered the following traits: note duration (ND, seconds), note modulation (NM, Hertz), interval between notes (IN, seconds); Lowest frequency (LF, Hertz), highest frequency (HF, Hertz), and peak frequency (PF, Hertz). Call measurements are illustrated in Fig. 3.

Our data set was checked for normality prior to the parametric statistical analyses. In order to assess the effect of temperature on call properties, we performed linear regression analyses for all populations combined. For all within- and among-population analyses, we used temperature-adjusted acoustic data by calculating overall regression coefficients (b) for all call parameters at the mean temperature of 25.9°C (SD= 1.77, range=23.1–31.2) through the equation $Y\,\mathrm{adj} = y - (b \times t_{\mathrm{calling\,site}}) + (b \times t_{\mathrm{mean}})$ where $Y\,\mathrm{adj}$=adjusted value of call measurement, b=regression coefficient, and t=temperature. The relationship between temperature-adjusted acoustic traits and male body size was also assessed by linear regression analyses. The adjusted r^2 values obtained were used to estimate the percentage of variation explained by the independent variable.

Coefficients of variation ($CV = SD \times 100/\mathrm{mean}$) for each call trait were calculated within populations (CVwp), within species (CVws), and over all individuals analyzed (CVo). Our objectives were to evaluate the degree of variability of each call measurement and to determine which call parameters might be most distinctive among populations
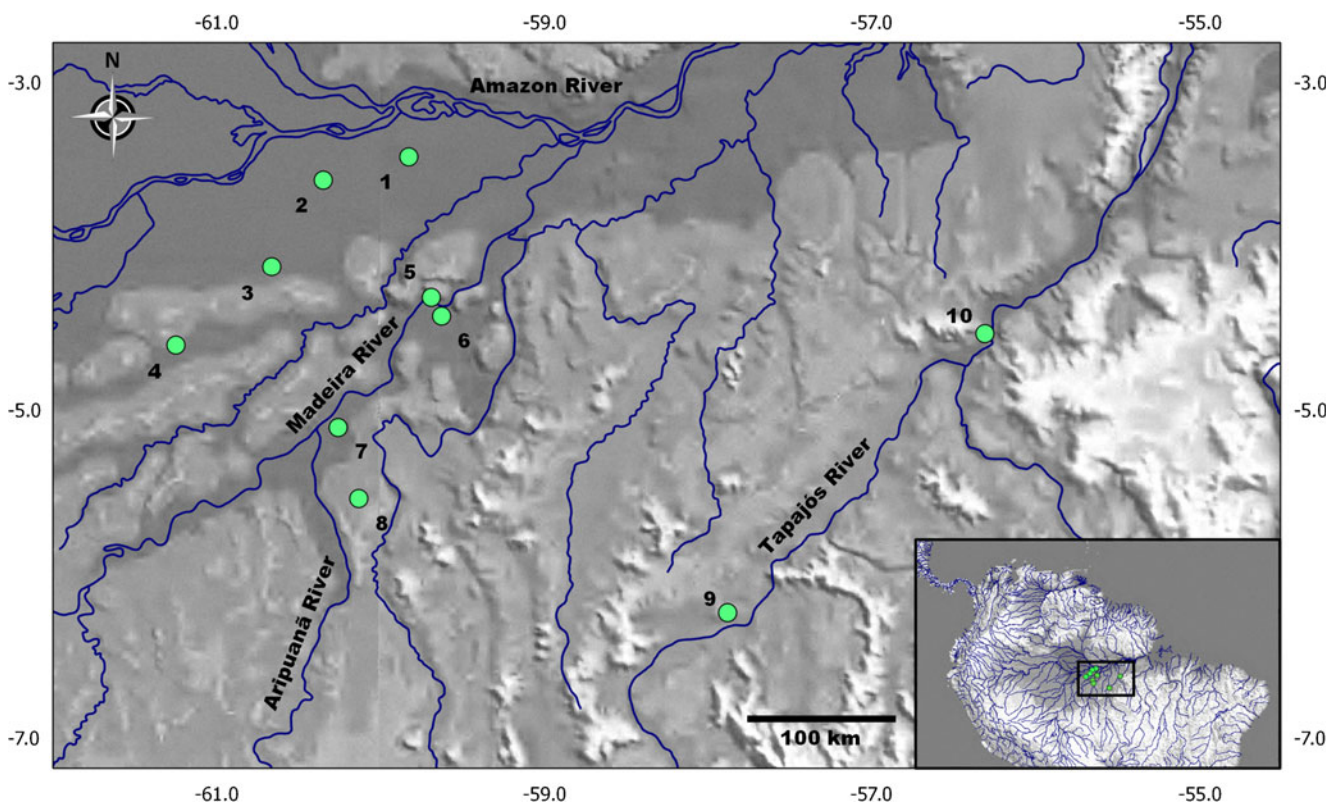
Fig. 2 Sampling sites visited in this study: *1* Km 12 of Autazes Road, type locality of *A. nidicola*; *2* BR-319, km 260; *3* PPBio Manaquiri; *4* BR-319 Tupana; *5* Vila Gomes; *6* Borba; *7* Novo Aripuanã; *8* Road to Apuí; *9* Jacareacanga; *10* PARNA da Amazônia, type locality of *A. masniger*

Fig. 3 A graphical representation of the call measurements adopted in this study. Oscillogram (**a**) and spectrogram (**b**) of a call sequence of a topotypic *A. masniger* male (SVL= 18.5 mm; INPA-H 28196), recorded at air temperature of 23.7°C. Power spectrum (**c**) of a single call of this individual. Highest and lowest frequencies were measured at 20 dB below the peak intensity (*horizontal dotted line*), the value at which the signal energy could still be clearly distinguished from background noise. Note modulation was calculated as the difference between the highest and the lowest frequencies of the call
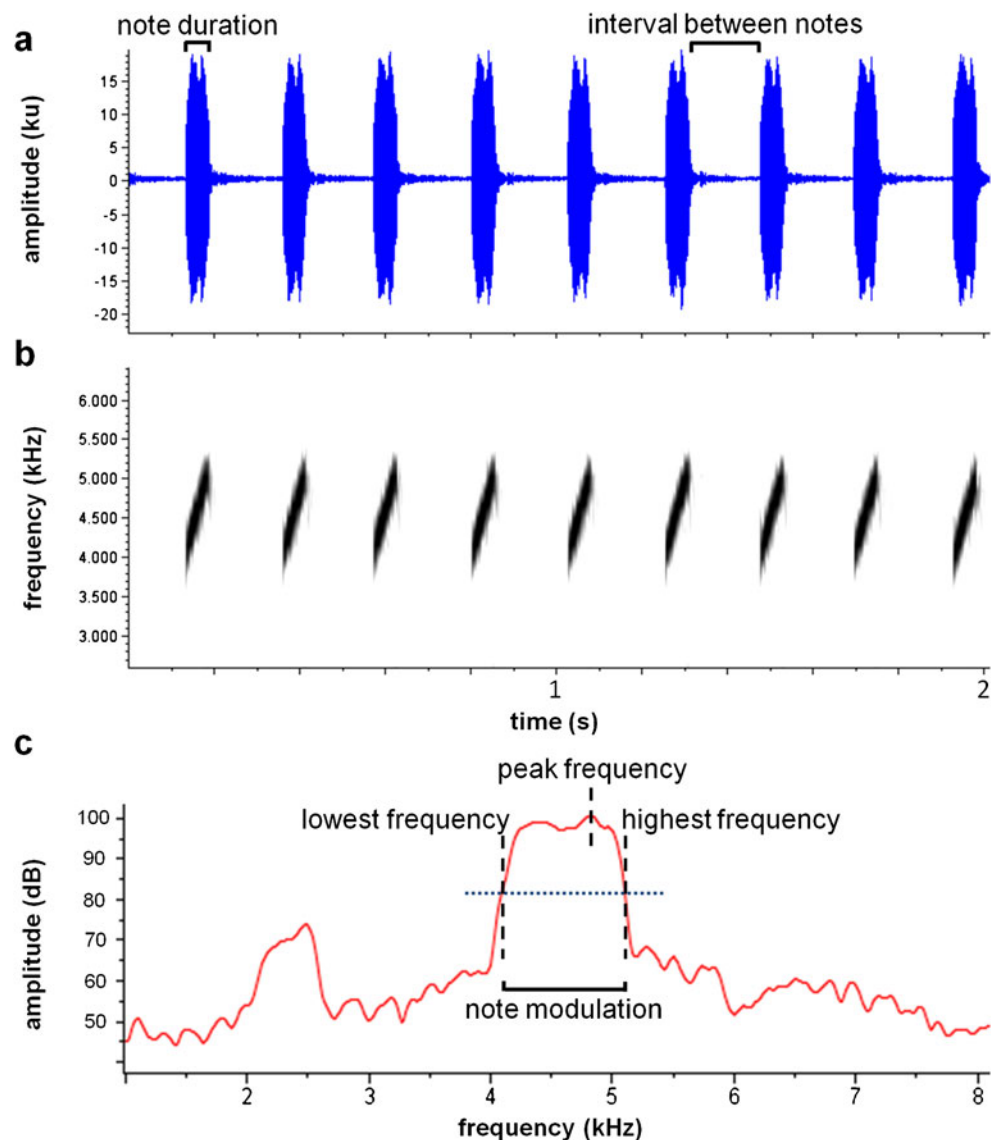
and between species. Within-population CVs are based on the mean and SD over all call-recorded individuals in each population ($n=10$). We obtained the mean within-population CV (CVwp) by averaging all within-population CVs. Within-species CVs are based on the mean and SD over all call-recorded individuals in each side of the river. We obtained the mean within-species CV (CVws) by averaging the two within-species CVs. Overall CVs (CVo) were obtained from the grand mean and SD over all populations and were initially calculated for individuals ($n=100$) of all populations combined (Pröhl et al. 2007).

To compare the difference in calls (with temperature-adjusted traits) between opposite sides of the Madeira River (species) and among populations, we conducted nested analyses of variance (ANOVA), with populations nested in river sides. Two different models for each call trait were used: (a) a nested ANOVA without cofactor and (b) a nested ANOVA with size as cofactor (nested ANCOVA).

These approaches allowed us to compare the *F* statistics obtained through the inclusion of body size as a cofactor in the analyses: if an interaction takes place between cofactors and populations, a decline in *F* statistics is expected due to worsening of the model.

The correlation between linear geographical/body size distances among sampling sites and the respective acoustic distances was evaluated through Mantel tests (Smouse et al. 1986). A body size distance matrix was obtained through the calculation of Euclidean distances between mean SVL measurements for each sampling locality. The acoustic distance matrix was calculated by conducting a principal components analysis from the data set of temperature-adjusted call measurements. We obtained pairwise Euclidean distances between all possible pairs of populations by using the scores of the mean acoustic measurements in the first and second principal components (which together explained 71.4 % of the total acoustic variation). Mantel tests were

Fig. 4 Within-population coefficients of variation (CV, in percent) for six acoustic properties of the advertisement call of the individuals studied. *Gray boxes* indicate the middle 50 % of the data (interquartile range), and *horizontal lines* represent the median values. *Vertical lines* indicate the 10th and 90th percentiles. Note that the lowest coefficients of variation are related to spectral traits of the call
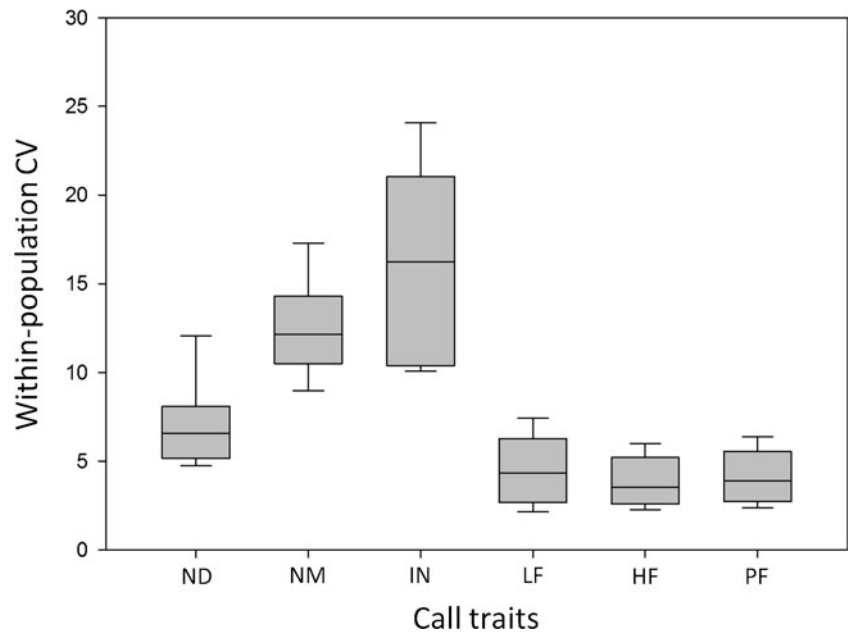
conducted in ZT (Bonnet and van de Peer 2002), using permutation of the null models (Anderson and Legendre 1999), and applying 10,000 randomizations.

Results

The coefficients of variation, which indicate the degree of variability of the call traits, differed when within-population, within-species, and overall variability were considered. Considering the within-species and overall perspectives, most of the call traits analyzed showed intermediate coefficients of variation. Regardless of grouping, spectral acoustic traits were the less variable (Fig. 4). They also showed the highest CVo/CVwp and CVo/CVws ratios, which suggests that spectral characters are more distinctive than temporal traits among populations and species (Table 1).

Environmental temperature significantly affected all call measurements except note modulation. Body size induced variation of all spectral properties of the call (Table 2). Acoustic traits differed statistically among the populations. The *F* statistics of spectral trait differentiation decreased when body size was included as a covariate. However, among-population differentiation was still significant for the highest frequency after this procedure. The lower Madeira River showed a strong effect on acoustic differentiation for all acoustic traits considered, even when the effects of male body size were controlled (Table 3).

When all localities sampled were considered, the Mantel test revealed a strong association between the advertisement call and body size differences between population pairs. Geographical distances were not correlated with acoustic or body size distances. When the Mantel tests were

Table 1 Descriptive statistics of the call traits analyzed, based on mean individual call properties of 100 individual males, adjusted to 25.9°C using linear regression

Trait	Mean±SD; *A. nidicola*	Mean±SD; *A. masniger*	CVwp	CVws	CVo	CVo/CVwp	CVo/CVws
ND (s)	0.048±0.005	0.050±0.005	7.1±2.3	9.4±0.6	9.6	1.36	1.01
NM (Hz)	354.7±46.7	396.9±51.8	12.5±2.6	13.1±0.1	14.2	1.14	1.08
IN (s)	0.290±0.048	0.339±0.086	16.2±5.4	20.9±6.0	23.3	1.44	1.11
LF (Hz)	4048.9±255.6	4299.3±231.1	4.5±1.9	5.8±0.6	6.5	1.45	1.12
HF (Hz)	4403.9±253.7	4699.4±207.2	3.8±1.3	5.0±0.9	6.0	1.58	1.18
PF (Hz)	4222.5±244.3	4497.6±209.7	4.0±1.4	5.2±0.8	6.1	1.50	1.16

Within-population (CVwp), within-species (CVws), and overall (CVo) coefficients of variation (percent, given as mean±SD) of six traits of the advertisement call of *A. nidicola* and *A. masniger*

Table 2 The effect of temperature and body size on call traits of the individuals studied ($n=100$ males)

Trait	Temperature			Body size		
	r^2	F	p	r^2	F	p
Note duration	0.45	82.00	<0.001*	−0.003	0.62	0.562
Note modulation	−0.003	0.69	0.589	−0.01	0.00	0.985
Internote interval	0.07	8.62	0.004*	0.03	3.60	0.057
Lowest frequency	0.07	8.15	0.005*	0.41	69.64	<0.001*
Highest frequency	0.06	7.84	0.006*	0.43	74.59	<0.001*
Peak frequency	0.07	8.61	0.004*	0.42	73.10	<0.001*

Adjusted linear regression coefficients of determination (r^2), F statistics, and significance values (p) are presented. For body size, statistics were calculated using temperature-adjusted call traits. Significant p values after Bonferroni adjustment are marked with asterisks

restricted to the left and right sides of the Madeira River (species), no correlation regarding the traits analyzed was found between population pairs (Table 4).

Discussion

What is the degree of variability of each of the call traits among populations and between species?

Coefficients of variation were higher in the overall perspective and reached its lower values within populations for all call traits analyzed (i.e., CVwp<CVws<CVo). Our results showed that spectral call properties were more distinctive than temporal traits among populations and species, with higher distinctiveness at the among-population level compared to the between-species level (i.e., CVo/CVws<CVo/CVwp). Therefore, our understanding of the effect of river barriers in speciation processes would be greatly benefited by future approaches that evaluate the degree of stereotypy of call traits at the within-individual level, as well as the role of spectral properties of the advertisement call in mate recognition and choice between individuals from opposite river sides.

To what extent do temperature and body size induce call trait variability?

In spite of the small range of variation in air temperature characteristic of tropical environments (23.1 to 31.2°C in this study), there was strong temperature-induced plasticity in call traits. Note duration had 45 % of its variation induced by this variable. Spectral characters of the call were also significantly affected by air temperature, although to a lesser extent (6–7 %). Significant effects on frequency-related call measurements were also observed in *Oophaga pumilio* throughout its range (Pröhl et al. 2007), but not in *Allobates femoralis* in a single locality (Gasser et al. 2009). As ectotherms, frogs likely have spectral call traits that are indirectly subject to the strong physiological constraints imposed by the environmental and consequently the body temperature (Prestwich 1994).

The effects of body size on the advertisement call of the individuals were restricted to frequency-related characters, as is most commonly reported for anurans (Gerhardt and Huber 2002). Body size-induced plasticity was pronounced in these measurements, ranging from 41 to 43%. Because of the invariant nature of these call properties, it was suggested that spectral characters of the advertisement call may be

Table 3 Nested analysis of (co-) variance of call traits (dependent variables) of *A. nidicola* and *A. masniger*

Population (nested in river side) and river side (species) are the dependent categorical variables. In model b, size is used as covariate. Significant p values after Bonferroni adjustment are marked with asterisks

Trait	Nested ANOVA				Nested ANCOVA (size)			
	F Pop	p	F River	p	F Pop	p	F River	p
ND	8.62	<0.001*	7.72	0.006*	8.67	<0.001*	7.49	0.007*
NM	1.34	0.232	18.79	<0.001*	1.30	0.249	24.08	<0.001*
IN	8.60	<0.001*	19.99	<0.001*	8.53	<0.001*	10.22	0.001*
LF	6.09	<0.001*	37.35	<0.001*	2.14	0.039	9.66	0.002*
HF	8.02	<0.001*	64.01	<0.001*	3.58	0.001*	22.41	<0.001*
PF	6.58	<0.001*	53.13	<0.001*	2.55	0.014	16.92	<0.001*

Table 4 Mantel tests evaluating correlations between acoustic (DAco), body size (DSVL), and geographical (DGeo) distances of *Allobates* sampled at ten localities in Central Amazonia

Model	r	p
All localities		
DAco×DGeo	−0.15	0.312
DAco×DSVL	0.642	0.001*
DSVL×DGeo	−0.193	0.205
Left side (*A. nidicola*)		
DAco×DGeo	−0.484	0.108
DAco×DSVL	0.409	0.191
DSVL×DGeo	0.152	0.366
Right side (*A. masniger*)		
DAco×DGeo	−0.355	0.166
DAco×DSVL	0.45	0.091
DSVL×DGeo	−0.419	0.058

Because of river effects, tests were also restricted to the left and right sides of the Madeira River (five localities each). Significant values after Bonferroni correction are marked with an asterisk

important indicators of male size (e.g., Bee et al. 2000; Smith and Hunter 2005). Body-size effects are frequently removed in comparative population analyses. However, as ontogenetic differences may be successfully controlled by this procedure, natural variability in adult SVL, which might be important and even diagnostic in comparative studies, is overlooked by applying body-size corrections (e.g., Castellano et al. 2000; Pröhl et al. 2007). Therefore, despite the difficulties in differentiating between the two sources of variability related to body size, the description of these relationships is important to the investigation of the morphological constraints of acoustic variation. Interestingly, the properties of note modulation and internote interval were little affected by temperature and the size of the frogs. This suggests that additional underlying factors, such as physiological state, body condition, and social environment (Castellano and Giacoma 1998; Tárano 2001; Wells and Schwartz 2007), might be responsible for the residual (i.e., unexplained) variability in the call traits analyzed.

Is the river barrier effect significant among all the different acoustic variables?

The river barrier effect was significant among all the different acoustic variables analyzed, even after the effects of male body size were controlled. The inclusion of SVL as a covariate lowered the *F* statistics obtained in the analysis of spectral measurements, suggesting an interactive effect between geography and body size on call differentiation. Our results in the nested analysis of variance complement the findings of Tsuji-Nishikido et al. (2012) by addressing the

role of the lower Madeira River in affecting each of the call traits. The effect of Amazonian rivers has been observed in a range of organisms, evidencing its important role in evolutionary processes in the biome (e.g., Ayres and Clutton-Brock 1992; Peres et al. 1996; Hayes and Sewlal 2004; Simões 2010). In contrast, the among-population differentiation regarding call traits was severely reduced after we accounted for body-size effects and was restricted to the properties of note duration, interval between notes, and highest frequency. Thus, the overall ANOVA results show a predominant effect of the Madeira River in addition to the among-population differences in the acoustic signals studied.

Does isolation by distance play a significant role in call divergence beyond the known effects of the river as a barrier?

No correlation between geographic distance (a potential predictor of both degree of environmental similarity and gene flow) and acoustic (sexual signal) dissimilarities was revealed by the Mantel tests, and the observed pattern held when the analyses were restricted to each side of the river. This apparent absence of isolation-by-distance effects was also observed for call differentiation in *Allobates paleovarzensis* in Central Amazonia (Kaefer and Lima 2012), but not for the neotropical frogs *Dendropsophus leucophyllatus* and *A. femoralis* on wider geographical scales (Lougheed et al. 2006; Amézquita et al. 2009). This suggests that local stabilizing selective pressures may be more important than neutral effects, such as genetic drift, in the evolution of call differentiation in the *A. nidicola/masniger* system. Regarding the two geographical factors presumed to be driving phenotypic differentiation among the populations studied—river and linear geographical distance—our results give further support to the relationship between sexual signal differentiation and the Madeira River as a barrier, as indicated by Tsuji-Nishikido et al. (2012). A molecular assessment of population variability, together with data on phenotypic variation, will seek to assess the relationship between genetic and phenotypic divergence between *A. nidicola* and *A. masniger*, a study system that shows the potential to elucidate various aspects regarding the initial stages of allopatric speciation.

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