Zoologischer Anzeiger 268 (2017) 143–150

Contents lists available at ScienceDirect

Zoologischer Anzeiger

journal homepage: www.elsevier.com/locate/jcz

Zoologischer Anzeiger

Research paper

Call variability, stereotypy and relationships in syntopy of tetraploid common lesser escuerzo (Anura: Genus *Odontophrynus*)

Pablo R. Grenat[a,b], Julián A. Valetti[a,b], Adolfo L. Martino[a,*]

[a] *Ecología, Departamento de Ciencias Naturales, Facultad de Ciencias Exactas, Físico—Químicas y Naturales, Universidad Nacional de Río Cuarto, Ruta Nacional N° 36-km. 601, X5804BYA Río Cuarto, Argentina*
[b] *CONICET, Argentina*

ARTICLE INFO

Article history:
Received 4 December 2015
Received in revised form 21 June 2016
Accepted 23 June 2016
Available online 25 June 2016
Corresponding Editor: Alexander Kupfer

Keywords:
Advertisement call
Anuran communication
Bioacoustics
Character displacement
Geographic variation
Odontophrynus
Syntopy

ABSTRACT

We studied patterns of the geographic variation of mating calls of the common lesser escuerzo *Odontophrynus americanus* including populations living in syntopy with the closely related *O. cordobae* and adjacent allopatric populations. The dominant frequency, pulse rate, call duration and pulses/call showed the lowest intra-individual variation (CV < 3%). The discriminant function analysis (DFA) assigned 88% of all calls to the correct individual, indicating that the advertisement calls of *O. americanus* are individually distinctive. Call duration, pulses per call and the dominant frequency were likely related to individual distinctiveness. Pulse rate and pulse temporal parameters related consistently showed the lowest values of CV_b/CV_w ratio and could be considered key properties for species recognition in *O. americanus*. We found a greater call divergence between species in syntopy than in allopatry in terms of the pulse rate and particularly the interpulse interval. These results appear to be consistent with a pattern of reproductive character displacement. Further studies testing female selectivity should be essential to evaluate whether acoustic differences observed between syntopic and allopatric populations are biologically significant and if there are differences in selectivity between such areas.

1. Introduction

Animal signals can vary at different scales and the identification and characterization of this variation is a critical first step in the understanding of the adaptive functions of signals (Gerhardt and Huber, 2002; Bee et al., 2010). In anurans there are many studies that document intra-specific variation in frog calls, measuring and comparing variation within and among individuals in a population (e.g., Tarano, 2001; Castellano et al., 2002; Smith and Hunter, 2005; Grenat et al., 2013). Furthermore, several intra-specific studies demonstrated that inter-populational differences in sexual signals are common and influence mate recognition (Pröhl et al., 2006, 2007; Dreher and Pröhl, 2014). However, the implications of the variability in the expression of sexual signals are still poorly understood (Smith and Hunter, 2005). Identifying potential causes of pattern of divergence in characters within and among populations provides support for hypotheses about how divergence arises and how it is maintained (Jang and Gerhardt, 2006). Consequently, the analysis of signal variation at different levels of organization might provide an important source of information for the understanding of signal evolution (Castellano et al., 2002b; Gasser et al., 2009). Additionally, geographic overlap with other related species can not only promote acoustic divergence between species, and between allopatric and sympatric populations of a single species, but it can even lead to differentiation of calls and preferences among conspecific populations within the contact zone (Gerhardt, 1994; Higgie et al., 2000; Lemmon, 2009; Gerhardt, 2013).

Tetraploid *Odontophrynus americanus* (Duméril and Bibron, 1841) was the first natural bisexual polyploid anuran species described (Beçak et al., 1966). In central Argentina, it is distributed to the south of the province of Córdoba and the northernmost populations coexist in syntopy with their cryptic related species, the diploid *Odontophrynus cordobae* (Martino and Sinsch, 2002; Grenat et al., 2009a,b). As individual differences and geographic variation of advertisement calls between syntopic and non-syntopic populations of *O. cordobae* have been found (Grenat et al., 2013) it would be highly interesting to know whether similar call patterns are present in the closely related polyploid *O. americanus*.

In this paper, we examine call trait variation and stereotypy of the common lesser escuerzo *O. americanus* in central Argentina. We characterized the acoustic properties on the basis of their intra- and

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* Corresponding author.
E-mail addresses: adolfomartino@gmail.com, amartino@exa.unrc.edu.ar (A.L. Martino).

http://dx.doi.org/10.1016/j.jcz.2016.06.007
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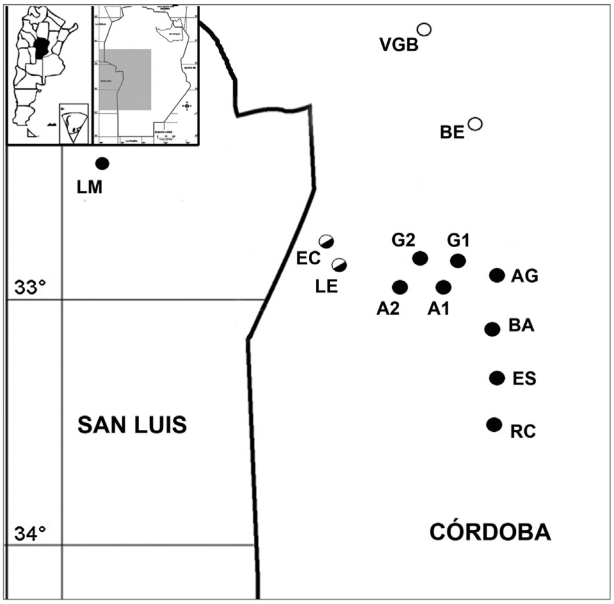

Fig. 1. Geographical position of sampled *Odontophrynus americanus* (black point) and *O. cordobae* (white point) populations. LM, Los Membrillos; RC, Río Cuarto; BA, Baigorria; ES, Espinillo; AG, Alcira Gigena; A1, Alpa Corral-La Escondida S1; A2, Alpa Corral-La Escondida S2; G1, Gigena-El Cano S1; G2, Gigena-El Cano S2; EC, El Cano; LE, La Escondida; BE, Berrotarán; VGB, Villa General Belgrano.

interpopulational variation and we studied patterns of geographic variation of *O. americanus* advertisement calls. Finally, we evaluated call differences between *O. americanus* and *O. cordobae* in allopatric and syntopic populations and we analyzed the directionality of call change in both species living syntopically.

2. Materials and methods

2.1. Sampling and study site

The anuran *Odontophrynus americanus* is a prolonged breeder, which reproduces during austral spring-summer months (September–March) using temporary and permanent ponds. We recorded advertisement calls of individuals between 1999 and 2012 from ponds in 11 sampling sites from the provinces Córdoba and San Luis: Los Membrillos, Río Cuarto, Baigorria, Espinillo, Alcira Gigena, Alpa Corral-La Escondida S1, Alpa Corral-La Escondida S2, Gigena-El Cano S1, Gigena-El Cano S2, El Cano, La Escondida (Fig. 1). In the last two sites, populations of tetraploid *O. americanus* coexist in syntopy with its cryptic diploid counterpart, *O. cordobae* (Grenat et al., 2009a,b) while the remaining study sites were considered allopatric because no *O. cordobae* were found. We did not include more distant sites across the species' distribution range because our focus was to study the short scale variation. For the analysis between species, we include calls of *O. cordobae* from syntopic (La Escondida) and allopatric (Berrotarán; Villa General Belgrano) populations (Grenat et al., 2013).

2.2. Recording and analysis of advertisement calls

Vocal series of three to ten advertisement calls were recorded from 130 *O. americanus* and 42 *O. cordobae* males using a Walkman Digital AudioTape (DAT) Sony^{TM} TCD-100 with stereo microphone ECM-MS907 Sony^{TM}. Throughout each recording, the microphone was held at around 1 m from the focal calling male. Immediately after the acoustic recording, water temperature of breeding site (to the nearest 0.1 °C) was registered. These temperatures ranged between 15.1 °C and 28 °C.

Because these species are morphologically cryptic, the ploidy of individuals was analyzed by mean erythrometry in all populations for a correct identification of the species (see Grenat et al., 2009a,b). In syntopic populations cytogenetic analyses of males sampled acoustically were performed for ploidy confirmation (Grenat et al., 2009a,b; Otero et al., 2013). *Odontophrynus americanus* is very sensitive to disturbance and often disrupted its acoustic activity for long periods when it feels threatened. Therefore, it was not possible to associate the body measurement of every single calling male because, in many cases, males were not captured until after recording the call series of all males at the sample site. As several authors have reported a relationship between body size and the spectral call properties, we conducted a separate intra- and inter populational analysis to evaluate the morphometric variation. We obtained very low intra-population variability in body length (SVL) with coefficients of variation (CV) of less than 9%. In addition, no significant differences between populations in terms of SVL were found (ANOVA, $p = 0.0543$). These results agree with a previous morphometric study including the same sites (see Grenat et al., 2012).

Acoustic signals were digitized and analyzed by means of oscillograms, spectrograms and power spectra using the software Adobe® Audition^{TM} 1.0 (sampling rate: 44.1 KHz; bit depth: 16 bit). A call of *O. americanus* consists of a single pulse group, with pulses located at the beginning of the call having low intensity, followed by increasing intensity pulses, and a decrease in pulse intensity at the end of call. The call structure of *O. americanus* is similar to *O. cordobae* (Fig. 2). Frequency spectra were calculated with the following settings: window function hamming, frame length 512 points, grid resolution 128 points, FFT size 1024 points; amplitude logarithmic. Each call series was characterized by six call properties: (1) Call duration (CD) [ms]; (2) Pulses/Call (P/C) [N]; (3) Pulse duration (PD) [ms]; (4) Interpulse interval (IPI) [ms]; (5) Intercall interval (ICI) and (6) Dominant frequency (DF) [Hz]. Pulse rate (PR) [pulses/s] and Duty cycle (DC) [PD/IPI] were also calculated (Martino and Sinsch, 2002; Grenat et al., 2013). Call properties associated with pulse characteristics (e.g., PD and IPI) were measured in at least three pulses in each call.

2.3. Statistical analyses

The arithmetic means of all call parameters were calculated for each series and used for further analyses. Acoustic data were shown to be normally distributed using the Shapiro-Wilk test for normality ($p < 0.05$). Linear regressions between call variables and water temperature were performed to assess the effect of this environmental variable on acoustic traits. Because temperature can affect anuran call properties (Gerhardt and Huber, 2002), we corrected all properties which were significantly correlated with temperature by adjusting their values to 20 °C (mean of registered temperatures) following Heyer and Reid (2003).

2.3.1. Variability in call structure of O. americanus at different levels

We used a coefficient of variation ($CV = (SD/Mean) \times 100$) to compare patterns of variation at different levels: within individual (among calls from the same individual), within population (among individuals from the same population) and among populations. CVs of higher levels were estimated using the mean values of the acoustic parameter obtained at the immediately lower level, applying an approach similar to that employed in the variance computation in a nested ANOVA model (see Castellano and Giacoma, 1998). We used within-male CVs to characterize the variability of each call properties as static ($CV_w < 4\%$) or dynamic ($CV_w > 12\%$), following Gerhardt (1991). We calculated the ratio of between-male to within-male CV ($CV_b \not\subset V_w$) as a measure of relative between-male variability.

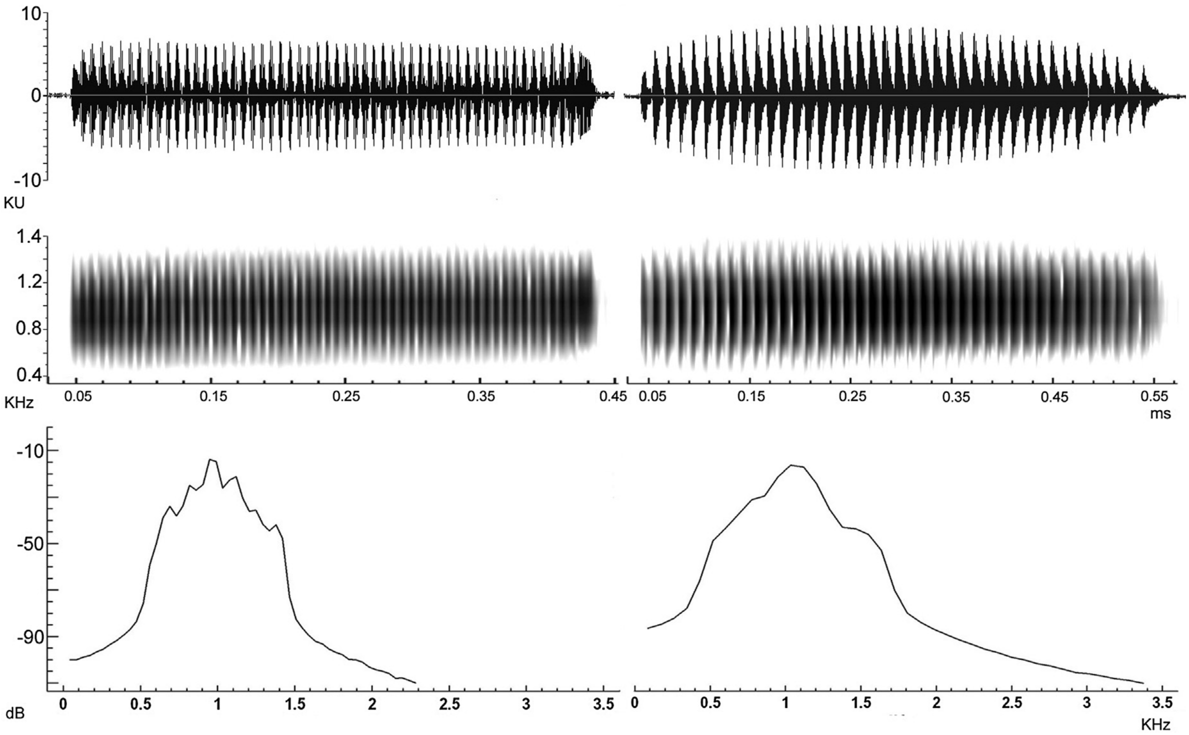

Fig. 2. Oscillogram, spectrogram and power spectrum of *Odontophrynus cordobae* (left) and *O. americanus* (right) advertisement call.

If the CV$_b$ ⊄ V$_w$ ratio was >1.0 for a property, then the property is relatively more variable among individuals and may function as individual recognition cue (Bee et al., 2001; Gasser et al., 2009). To estimate whether the observed variability in call properties varied significantly among males, we conducted a one-way ANOVA (Gasser et al., 2009).

For call comparison between *O. americanus* populations, separate univariate analyses of variance (ANOVA) were performed for each call property. If significant differences were found, pairwise tests between populations (Tukey's HSD) were applied to determine which groups showed differences. Discriminant function analysis (DFA) was used to examine the individual and population distinctiveness when all call properties were included. For populational analysis we included populations with more of five individuals. The discriminant functions generated in a DFA can be used to classify signals as belonging to particular individuals. Furthermore, classification success serves as a measure of how well the linear combinations of variables in the discriminant functions distinguish among individuals (Bee et al., 2001). Phenetic clustering of populations based on acoustic distance (squared Mahalanobis distances) was applied using the unweighted pair-group method with arithmetic averaging (UPGMA).

2.3.2. *Geographic patterns of call variation*

To evaluate a potential clinal call variation in *O. americanus*, mean population values of temperature-adjusted call variables were regressed against latitude. To examine the geographic distance effect, we performed Mantel tests (MANTEL for Windows 1.18; Calvalcanti, 2008). This test calculates correlations among similarity/dissimilarity and distance matrices considering that the same data point is used for multiple comparisons. We built a Mahalanobis distance dissimilarity matrix between the means of call variables, and correlated it with a matrix of linear geographical distances between all pairs of study sites. Ten thousand iterations were used to assess the significance of the results.

2.3.3. *Call variation between species: syntopic versus allopatric populations*

For analysing acoustic behaviour in contact areas we included advertisement calls of syntopic and allopatric *O. americanus* and closely related *O. cordobae*. Only the more distant allopatric populations of *O. americanus* (i.e., Río Cuarto, Espinillo and Los Membrillos) in respect to the contact areas entered the analysis. In all analyses we considered four groups: allopatric *O. americanus*, syntopic *O. americanus*, allopatric *O. cordobae* and syntopic *O. cordobae*.

For acoustic comparisons between species we performed separate ANOVAs for each call parameter. Principal Components Analysis (PCA) was used to statistically examine the group distinctiveness when all call variables were included. To evaluate the magnitude of acoustic differences between *O. americanus* and *O. cordobae* allopatric and syntopic populations, we performed a discriminant function analysis (procedure: backward selection). Phenetic clustering (UPGMA) of groups based on acoustic distance was applied to examine the directionality and magnitude of the call divergence. All calculations were performed using the program package STATGRAPHICS for Windows, version 5.0, and INFOSTAT v.2013.

3. Results

3.1. *Variability in call structure of* O. americanus *at different levels*

Population means and standard deviations for all acoustic properties of *O. americanus* advertisement calls are given in Table 1. DF, PR, CD and P/C showed the lowest variation within-male (CV$_W$ < 4%), and can be considered as static properties (see Table 2). PD and IPI together with DC were classified as intermediate with values near to 5%. The intercall interval was considered the only dynamic property, showing CV$_W$ greater than 12%. CVs for properties classified as static or intermediates increased with the level of analysis both within individuals towards populations, while the

Table 1
Mean and standard deviation of acoustic measurements taken on call series of *Odontophrynus americanus* from 11 breeding sites and *O. cordobae* from three localities. Parameters abbreviations: CD: call duration; P/C: pulses/call; DF: dominant frequency; PD: pulse duration; IPI: interpulse interval; PR: pulse rate; DC: duty cycle. N: number of call analyzed.

Locality	N	Acoustical parameter						
		CD (ms)	P/C	PD (ms)	IPI (ms)	DF (Hz)	PR	DC
O. americanus								
Los Membrillos	21	462.1 ± 28.8	39.2 ± 2.7	5.5 ± 0.4	6.5 ± 0.4	869.5 ± 30.2	83.7 ± 3.6	0.86 ± 0.1
Río Cuarto	114	502.7 ± 81.1	39.1 ± 4.7	5.7 ± 0.6	7.6 ± 0.5	1051.3 ± 76.6	75.2 ± 4.1	0.76 ± 0.1
Espinillo	18	507.7 ± 43.8	41.0 ± 2.1	5.7 ± 0.8	6.8 ± 0.9	1033.9 ± 64.6	80.3 ± 6.2	0.8 ± 0.2
Baigorria	30	502.0 ± 31.5	42.7 ± 3.4	4.7 ± 0.5	6.2 ± 0.6	1033.0 ± 56.7	91.3 ± 2.0	0.77 ± 0.1
Alcira Gigena	54	530.9 ± 56.0	39.1 ± 4.8	5.0 ± 0.4	8.0 ± 1.1	1048.7 ± 130.6	80.4 ± 5.8	0.68 ± 0.1
Gigena-El Cano S1	15	496.7 ± 76.7	43.7 ± 6.1	5.2 ± 0.3	6.6 ± 0.6	962.3 ± 21.7	85.2 ± 3.9	0.81 ± 0.1
Gigena-El Cano S2	30	481.7 ± 45.3	40.2 ± 4.0	5.7 ± 0.4	6.3 ± 0.5	971.1 ± 47.7	82.9 ± 2.6	0.90 ± 0.1
A-Corral-La Esc S1	18	525.6 ± 31.1	45.2 ± 2.0	5.2 ± 0.3	6.2 ± 0.2	1003.0 ± 19.0	86.2 ± 1.9	0.9 ± 0.02
A-Corral-La Esc S2	12	481.3 ± 40.4	42.7 ± 4.5	5.7 ± 0.4	6.1 ± 0.4	1017.9 ± 20.5	86.2 ± 2.6	0.91 ± 0.1
La Escondida	39	435.9 ± 55.7	40.1 ± 3.1	4.9 ± 0.8	5.8 ± 0.9	963.6 ± 76.6	92.6 ± 10.8	0.87 ± 0.2
El Cano	21	540.1 ± 24.9	50.3 ± 2.2	5.1 ± 0.3	5.7 ± 0.3	1072.6 ± 26.8	92.1 ± 1.9	0.87 ± 0.1
O. cordobae								
V° Gral. Belgrano	90	432.2 ± 51.3	49.6 ± 3.4	3.9 ± 0.4	4.9 ± 0.5	990.7 ± 42.0	119.4 ± 3.7	0.85 ± 0.1
Berrotarán	60	412.9 ± 18.7	49.8 ± 2.2	3.8 ± 0.1	4.3 ± 0.2	927.8 ± 59.6	129.6 ± 4.2	0.87 ± 0.1
La Escondida	60	410.9 ± 49.8	52.2 ± 4.7	3.7 ± 0.2	3.8 ± 0.3	1007.3 ± 64.9	135 ± 9.1	0.98 ± 0.1

Table 2
Coefficients of variation for advertisement call parameters calculated at three different levels of analysis and CVa/CVw ratio for each acoustic property. CD: call duration; P/C: pulses/call; DF: dominant frequency; PD: pulse duration; IPI: interpulse interval; PR: pulse rate; DC: duty cycle; ICD: Intercall duration.

Analysis level	CD	P/C	DF	PD	IPI	PR	DC	ICD
Within individual	2.92 ± 2.04	2.90 ± 2.09	1.05 ± 1.22	4.31 ± 2.55	4.35 ± 2.60	2.25 ± 1.44	7.43 ± 4.49	18.62 ± 13.8
Among individuals	8.92 ± 3.05	8.74 ± 3.38	5.11 ± 3.23	8.41 ± 4.57	8.50 ± 3.35	4.88 ± 2.98	13.75 ± 5.43	40.23 ± 12.4
Among populations	11.29	12.55	8.63	12.36	14.18	10.96	16.8	42.49
CVa/CVw	3.05	3.01	4.87	1.95	1.95	2.17	1.85	2.16

dynamic call interval showed similar values within and among populations. All properties varied more among males than within males, with $\mathrm{CV_b}$ Ȼ $\mathrm{V_w}$ ratios higher than 1.8 (Table 2). Static properties exhibited higher $\mathrm{CV_b}$ Ȼ $\mathrm{V_w}$ ratios compared to both dynamic and intermediate properties (Table 2). Additional one-way ANOVA confirmed significant differences in the static properties between $\mathrm{CV_b}$ and $\mathrm{CV_w}$ ratios (ANOVA, CD: $F_{1,136} = 81.78$, $p < 0.0000$; P/C: $F_{1,136} = 73.40$, $p < 0.0000$; DF: $F_{1,136} = 81.50$, $p < 0.0000$; PR: $F_{1,136} = 28.65$, $p < 0.0000$). The $\mathrm{CV_b}/\mathrm{CV_w}$ ratio for the spectral property, dominant frequency, was considerably higher than for all temporal properties.

Individual acoustic distinctiveness was examined using discriminant function analysis. Discriminant functions were highly significant (Wilks' lambda = 0.00000059; canonical correlation = 0.995; $p < 0.0001$), and the first two functions explained 84% of the individual variation. The first function was strongly associated with P/C and CD, while the second function was mainly associated with the dominant frequency. DFA correctly classified 88% of the calls to a respective male.

Univariate analysis demonstrated significant differences among populations in all call parameters (CD: $F_{10,113} = 3.21$, $p < 0.01$; P/C: $F_{10,113} = 5.92$, $p < 0.0001$; DF: $F_{10,113} = 5.55$, $p < 0.0001$; PD: $F_{10,113} = 4.93$, $p < 0.0001$; IPI: $F_{10,113} = 16.35$, $p < 0.0001$; PR: $F_{10,113} = 19.13$, $p < 0.0001$; DC: $F_{10,113} = 4.27$, $p < 0.0001$). Pairwise tests between *O. americanus* populations showed that calls of syntopic populations were significantly different from the remaining populations in three out of seven acoustic properties (P/C, IPI, PR; Tukey test, $p < 0.05$). The result was supported by the UPGMA cluster analysis (Fig. 3). Herein the syntopic populations appeared as two distant groups separated of the remaining study populations.

When we grouped populations based on its acoustic characteristics using the discriminant function analysis, we obtained seven discriminant functions, the first four of which were highly significant. Functions 1 (Variance = 47%; Wilks' lambda = 0.0456745; canonical correlation = 0.84561; $p < 0.0001$) and 2 (Variance = 38%; Wilks' lambda = 0.160293; canonical correlation = 0.81820; $p < 0.0001$) had eigenvalues greater than 1 and accounted for 85% of the total variation. The first function was strongly related with call duration (standardized coefficient = −2.26761) and pulses/call (sc = 2.4685) while the second function loaded heavily with pulse rate (sc = −2.78446). DFA revealed a more pronounced separation of syntopic individuals with respect to the remaining populations that showed overlap each other (Fig. 4). However, in the classification of individuals on the basis of these discriminant functions, 77% (range 50–100%) were correctly assigned to their original population.

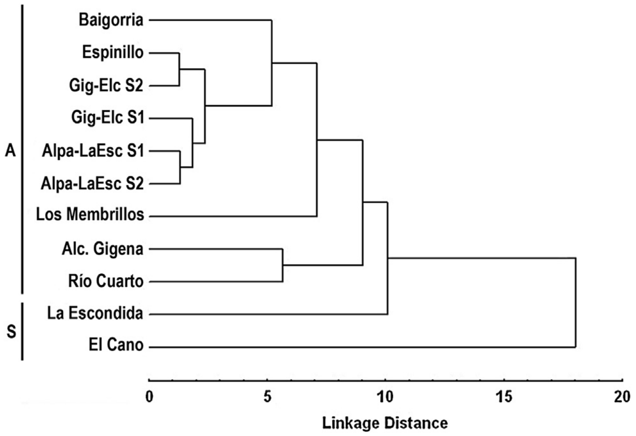

Fig. 3. Cluster analysis based on square Mahalanobis distances showing the similar acoustical population groups in *Odontophrynus americanus*. A: Allopatric; S: Syntopic.

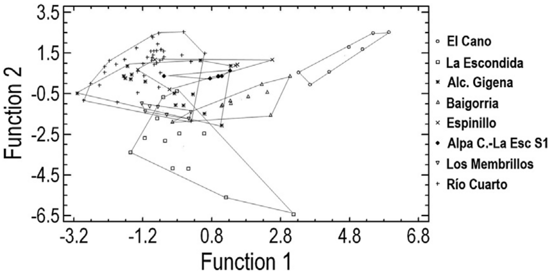

Fig. 4. Acoustic distinction among populations of *O. americanus* based on Discriminant Function Analysis.

3.2. *Geographic patterns of call variation*

Simple regressions between *O. americanus* call variables and latitude coordinates showed a north-south-wise increase in call duration ($r = -0.25$; $p < 0.01$), pulse duration ($r = -0.37$; $p < 0.0001$) and interpulse interval ($r = -0.48$; $p < 0.0001$) and a north-south decrease in pulse rate ($r = 0.63$; $p < 0.0001$) and pulses/call ($r = 0.32$; $p < 0.001$). Dominant frequencies and duty cycles were not significantly correlated with latitude (DF: $r = -0.15$; $p = 0.0859$; DC: $r = 0.015$; $p = 0.0979$). The Mantel test revealed no significant correlation between geographic and call distance (correlation matrix was $r = 0.101$ and the one-tail probability was p [random Z < observed Z] = 0.671).

3.3. *Call variation between species: syntopic versus allopatric populations*

In the univariate inter-specific comparison we found acoustical differences between syntopic populations in all call properties (ANOVA: $p < 0.001$) except in DF ($F_{1,29} = 0.12$; $p = 0.7301$) while non-syntopic populations differed significantly in all call traits ($p < 0.05$). In both comparisons CD, PD and IPI showed lower values for *O. cordobae* while P/C, PR and DC showed lower values for *O. americanus*.

The first three components of the principal component analysis explained 89% of the total variation. PC1 (eigenvalue = 4.09) significantly correlated with pulse rate and interpulse interval (component weight = 0.485 and 0.484 respectively), and was the key component separating the four groups: allopatric and syntopic populations of each species (Fig. 5). Discriminant function analysis was significant (Wilks' lambda = 0.033; canonical correlation = 0.97; $p < 0.0001$) and only three properties were maintained in the analysis (IPI, PR and DC). The first canonical function explained 92% of the total variation among the four groups (allopatric and syntopic populations of each species) and was mainly associated to pulse rate (standardized coefficient = 1.44). This function separate two species and, partiality, syntopic and allopatric calls within-species (Fig. 5). The second function mainly separated syntopic individuals between species and syntopic and allopatric calls of both species and was strongly associated to interpulse interval and duty cycle (standardized coefficients = 3.22 and 2.16 respectively). When pooling all individuals, 87% were correctly assigned to their original group. All groups showed a correct classification of individuals higher than 75%. No correctly classified individuals of syntopic populations were assigned as belonging to any allopatric populations of the same species.

Cluster analysis using squared Mahalanobis distances revealed two inter- and intraspecifically well-separated groups (Fig. 6). Interspecific analysis showed higher acoustic divergence between syntopic populations in comparison with allopatric populations (Fig. 6A and B). Intraspecifically, a higher acoustic differentiation was observed between allopatric and syntopic populations of *Odontophrynus cordobae* (Fig. 6C).

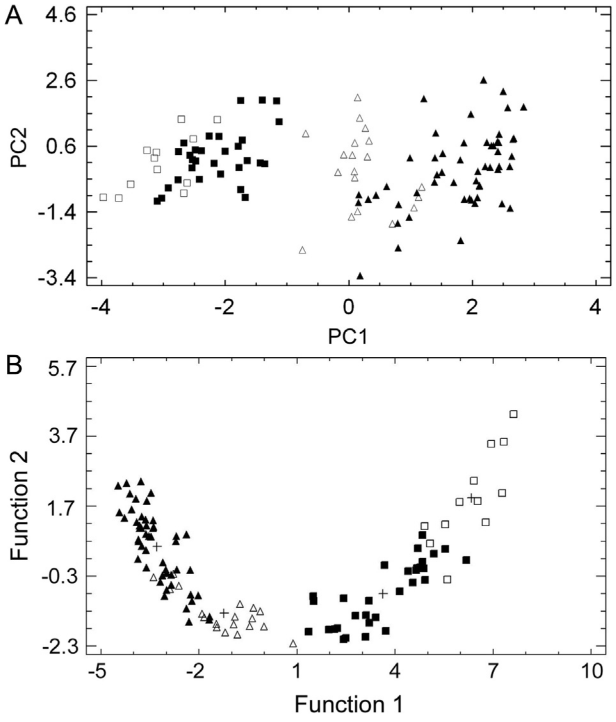

Fig. 5. Acoustic distinction among allopatric (fill) and syntopic (empty) populations of *O. cordobae* (square) and *O. americanus* (triangle): (A) Principal Component Analysis; (B) Discriminant Function Analysis.

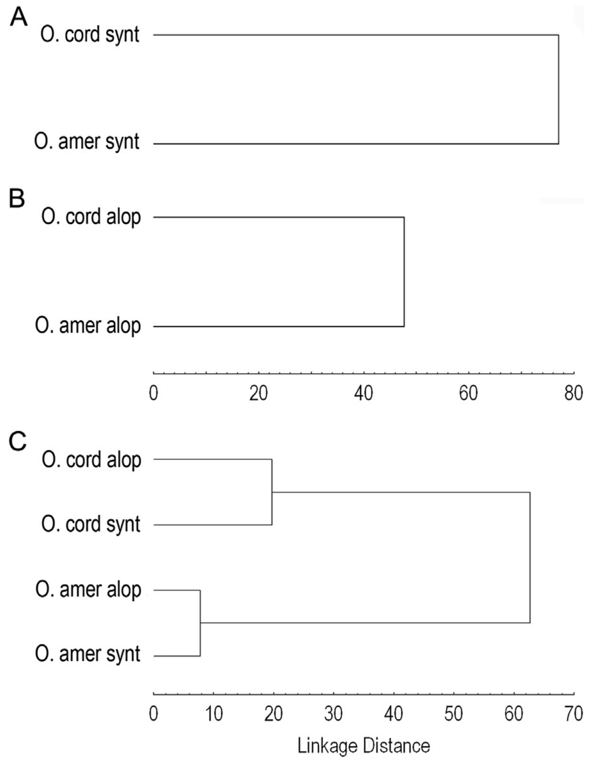

Fig. 6. Cluster analysis based on square Mahalanobis distances showing the acoustical association between population groups of *Odontophrynus americanus* and *O. cordobae*: (A) syntopic populations; (B) allopatric populations; (C) syntopic and allopatric populations of both species.

4. Discussion

4.1. *Patterns of call variability: dynamics and statics properties*

Static and dynamic properties have a potential to encode different kinds of biologically significant information: static properties might encode species, population or even individual identity, whereas dynamic properties might transmit mate-quality information (Gerhardt, 1992). Following Gerhardt (1991) we may categorize the PR, DF, CD and P/C as static properties and the ICI as dynamic trait for *Odontophrynus americanus*. As Gerhardt (1991) proposed, static and dynamic properties represent two ends of a continuum of variability. In support of this view, PD, IPI and DC were intermediately variable properties in our study. However, static or dynamic classification for a same property varies considerably across species. For example, call duration has been classified in different studies as static (Gerhardt, 1991; Friedl and Klump, 2002; Bee et al., 2010), dynamic (Gerhardt, 1991; Castellano and Giacoma, 1998; Gambale et al., 2014), or intermediate (Bee et al., 2001; Pröhl, 2003; Pettitt et al., 2013; Grenat et al., 2013). In general, relatively long signals exhibit greater intra-individual variability than shorter signals (Bee et al., 2010). Gerhardt (1991) suggests that this variability across species could be related to the temporal structure of the vocalization, with pulsed calls being more variable than unpulsed or very rapidly pulsed calls. Furthermore, since call duration depends upon the activity of muscles that force the air from the lungs through the larynx, the low intra-individual CVs for this parameter in a single calling bout might be caused by motor pattern stereotypy rather than morphological constraints (Castellano et al., 2002b). The number of pulses per call was also categorized as a static property but its low within-individual variation might arise from the combined effect of the low variation of call duration and the pulse/interpulse structure (Grenat et al., 2013).

The low within-individual variability of PR and DF and its consequent classification as static properties are consistent with previous studies in other anurans and insects (see Gerhardt and Huber, 2002). Such properties tend to be under stabilizing or weakly directional selection with females often preferring values at or near population means (Bee et al., 2010). Particularly, PR is reported as an important species recognition cue for many anurans (Loftus-Hills and Littlejohn, 1971; Schwartz, 1987; Schul and Bush, 2002) while DF is also proposed to be important in species recognition, but patterns of preferences for this parameter are more variable than those based on PR (Gerhardt, 1991). Unfortunately, our study fails to examine the possible relationship of the acoustic variables and body size. Spectral properties of most anuran advertisement calls are directly related to the frequency at which the vocal cords vibrate, and therefore depend upon their mass and tension, strongly associated with body size (Castellano and Giacoma, 1998). Our analysis of variability in SVL showed no intra or inter-population differences. This result is consistent with low intra- and inter-population variability observed in the DF and with the absence of correlation between this parameter and between latitude. Other authors have reported no association between SVL and DF in anuran species, suggesting that this call property does not provide receptive females with consistent information on the size of callers (*Pseudacris maculata* – Bee et al., 2010; *Anomaloglossus beebei* – Pettitt et al., 2013). However, our results are limited to infer whether the observed low variability is because there are forces acting directly on this acoustic property or is an indirect result of its association with body size.

On the other hand, dynamic properties may provide specific information to females about the energetic investment in courtship of potential mates (Gerhardt, 1991). Consequently, temporal parameters as intercall duration are constrained by the energy that males can invest in signalling and might therefore serve as a potential indicator of male relative condition (Castellano et al., 2002). It is known that ICI can change dramatically within a few seconds, usually in response to the behaviour of other conspecific individuals or external perturbations (Gerhardt, 1991; Castellano et al., 2002). Like many other species, *O. americanus* often breed in large groups or aggregations of signalling individuals. Several studies indicate that call alternation could reduce the masking of signals by conspecific or heterospecific neighbours (Brumm, 2006; Wong et al., 2009). Therefore, the observed variation in the intercall interval could be associated with strategies used for males to adjust their call cycles to coincide with periods of low masking noise.

4.2. *Individual distinctiveness of* O. americanus *advertisement call*

The DFA revealed that *O. americanus* advertisement calls are highly individually distinctive, with 88% of calls successfully assigned to the correct individual using a sample of 130 males. Other studies using multivariate approaches in anurans (e.g.; Bee et al., 2001, 2010; Gasser et al., 2009; Pettitt et al., 2013) and in other taxonomic groups (mammals – Blumstein and Munos, 2005; Charlton et al., 2011; birds – Jouventin et al., 1999; Reers et al., 2011; fish – Amorim et al., 2011) have demonstrated that individual distinctiveness in animal vocalizations is not unusual. These studies have reported discriminant function models typically assigning calls to the correct individual at rates between 50 and 100%. It should be noted that some variation among studies could result from differences in sample size included in the analysis (Bee et al., 2001; Gasser et al., 2009).

Two temporal parameters (CD and P/C), followed by the single spectral property (DF) contributed more to the individual distinctiveness in *O. americanus*. For a signal property to function as a useful cue for individual recognition, it must minimally show greater variation among individuals than within individuals (Jouventin et al., 1999; Bee et al., 2001; Pettitt et al., 2013). While all variables showed $CV_b \not\subset V_w > 1$, DF, CD and P/C had the highest $CV_b \not\subset V_w$ ratios (Table 2), accentuating their potential role as individual recognition cues. Although these properties showed a very low within-individual variability, among-individual variation was by far less stereotyped. Thus, these static properties could convey mainly information associated with individual distinction rather than the recognition of species.

Other studies have reported acoustic temporal properties mainly contributing to the individual distinctiveness in anurans (e.g.; *Allobates femoralis* – Gasser et al., 2009; *A. beebei* – Pettitt et al., 2013; *Scinax constrictus* – Gambale et al., 2014). Studies in diplo/tetraploid complex *Hyla chrysoscelis*/*H. versicolor* reported female preferences for call duration in both species highly directional in favour of calls with higher pulse numbers (Bee, 2008; Gerhardt et al., 2000) and indicated that *H. versicolor* females associated with this preference could receive indirect benefits in the form of increased offspring fitness (Welch et al., 1998; Welch, 2003). Hence, pulse number could be considered to be an honest indicator of a male's genetic quality or physiological condition (Bee, 2008).

Call properties may propagate differentially through the environment depending of habitat structure, calling site and background noise, and then a single call property may have a limited potential of identification for the signal receiver (Bee et al., 2001; Gasser et al., 2009). Sstereotyped temporal patterns could be useful for localization of conspecifics at close range and lower densities (Grafe, 1999; Bush et al., 2002). Consequently, preferences for longer calls are reduced when signals are perceived in noisy environments (Schwartz et al., 2001). On the other hand, low-frequency spectral components, such as dominant frequency of *O. americanus*, propagate well through breeding habitats (Bee et al., 2001) and could be mainly important for individual distinctiveness in large groups or aggregations of signalling individuals. For this, in many cases it would be expected males using a subset of call properties rather than a single property for discrimination (Bee et al., 2001; Gasser et al., 2009). Future playback studies using synthetic calls that vary in different properties could elucidate the potential role these properties play in individual discrimination for *O. americanus*.

4.3. *Species recognition traits of* O. americanus *advertisement call*

Some static properties may fail to uniquely identify individuals, not because they are variable within males, but because of limited among-male variability (Bee et al., 2001). For static properties, a low among-individual variation suggests that there has been a response to stabilizing selection and such traits are suitable for promoting species recognition (Gerhardt, 1991). PR and temporal parameters related (PD and IPI) consistently showed the lowest values of CV_b/CV_w ratio and could be considered key properties for species recognition in *O. americanus*. These acoustic properties were also reported as important for species recognition in the closely related *O. cordobae* (Grenat et al., 2013).

Signal properties under morpho-physiological constraints might show the lowest variation at the within-individual level, but the highest variation at micro- and macro-geographic scales, and they may convey key information for mate recognition (Castellano et al., 2002). PR showed a moderate clinal north-south decrease consistent with the opposite behaviour of PD and PI. The pattern of geographic variation observed was consistent with the higher variability obtained for among-population CVs in these acoustic parameters. Thus, these acoustic properties with greater restrictions could be the most appropriate to transmit messages whose misinterpretation would have a higher cost in fitness, for both signaller and receiver, and, at the same time, may provide the strongest responses to selection favouring the divergence of populations and, eventually, the origin of new species (Castellano et al., 2002).

For many anurans, pulse rate is a relevant call property used by females in selecting conspecifics mates, especially among closely related species that live in sympatry (e.g.; Schul and Bush, 2002; Lemmon, 2009). Furthermore, this call parameter has been commonly used for the interspecific distinction of closely related anurans belonging to diploid/polyploid or polyploid/polyploid complexes (e.g., *Hyla chrisoscelis*/*H. versicolor* – Ralin, 1977; *Bufo viridis* complex – Stöck, 1998; *Pleurodema kriegi*/*P. cordobae* – Valetti et al., 2009), including the diplo/tetraploid species pair *O. cordobae* and *O. americanus* (Martino and Sinsch, 2002). This acoustic difference between ploidy levels could be associated to a direct effect of polyploid on call structure, mainly on pulse rate, as result of increased gene dosage, ploidy specific alteration in gene expression, increased nuclear and cell volume or a combination of these factors (Ueda, 1993; Keller and Gerhardt, 2001) and the subsequent genotypic evolution increasing the divergence. In consequence, if the polyploidization had a direct effect on pulse rate the difference in this property could be sufficient for rejection of tetraploid calls by females of diploid species and vice verse (Keller and Gerhardt, 2001).

4.4. *Acoustic divergence between* O. americanus *and* O. cordobae *in syntopy: evolution of reproductive character displacement*

Multivariate analysis showed a higher acoustic differentiation between species (DFA = 100% positive classification) and within species (75–100%) in PR and pulse structure compared to other call traits. Several studies have shown that geographic overlap with related species can promote call divergence between species and between allopatric and sympatric populations of a single species (Pfennig and Ryan, 2006). For *O. americanus*, DFA and cluster analysis considering all studied populations showed an evident divergence between the syntopic populations of La Escondida and El Cano with respect to the other populations (Figs. 3 and 4), although the call properties associated to this displacement appeared to be different (PR rate and P/C, respectively). However, PCA and DFA including both species showed that PR is the property mainly separating species and IPI and DC appear to be the property regulating the divergence between syntopic and allopatric populations in both species, although their effect was more pronounced in *O. cordobae*. The analysis of acoustic distances revealed a higher divergence between species in syntopy than in allopatry and confirmed a higher displacement in the diploid species. These outcomes appear to be consistent with a pattern of reproductive character displacement (RCD; Brown and Wilson, 1956; Howard, 1993). Gerhardt (2013) defined RCD as a geographical pattern in which mate-attracting signals, preferences or both differ more in areas of sympatry than in areas of allopatry. Several robust examples of divergence in anuran communication systems in sympatric areas are consistent with RCD (see Gerhardt and Huber, 2002; Gerhardt, 2013). This evolutionary pattern may result from at least two processes including selection against hybridization (reinforcement) or selection against signal interference by neighbours (Howard, 1993). At present, natural hybrids between *O. cordobae* and *O. americanus* have not been found. However, artificial crosses among *O. americanus* diploid and tetraploid individuals from Brazil show that reproductive isolation is based on premating barriers and does not result from a reduced viability of hybrids (Beçak et al., 1970). In both species, syntopic individuals showed an increase in PR and a decrease in IPI. Since these call properties could be related to species recognition in both species (Grenat et al., 2013; our results) an accentuation of differences in these parameters and female preferences between species in syntopy would be expected in order to prevent mismating and gamete wastage. However, while differences in PR between allopatric and syntopic populations within each species were similar (about 15%), the divergence in IPI was higher in *O. cordobae* (20%) than *O. americanus* (15%) explaining the higher observed displacement in the diploid species. Preference criteria in closely related species in which the signal differences are mainly quantitative suggests that the co-evolution of senders and receivers is probably not always stepwise and parallel (Gerhardt, 2005). Schul and Bush (2002) reported divergence in selectivity of females in diplo/tetraploid complex of treefrogs. Whereas selectivity of *H. chrysoscelis* females is based on pulse rate alone, in *H. versicolor* it is based on an evaluation of pulse/interpulse structure (Schul and Bush, 2002). Consequently, to confirm the occurrence of RCD it would be essential to test female selectivity in further studies to evaluate (1) whether acoustic differences observed between syntopic and allopatric populations are biologically significant, and (2) if there are differences in receiver selectivity between such areas.

Acknowledgments

We thank an anonymous reviewer and Alexander Kupfer for their helpful comments. Financial support was provided by SECyT-UNRC (Grant PPI 18/C416) and FONCyT (Grant PICT 0932-2012; PICT2533/14). The first and second authors thank CONICET-Argentina (National Scientific and Technical Research Council) for postgraduate fellowship granted. Our study was authorized by Environmental Secretary of Córdoba Government (A01/2013).

References

Amorim, M.C.P., Simões, J.M., Almada, V.C., Fonseca, P.J., 2011. Stereotypy and variation of the mating call in the Lusitanian toadfish, *Halobatrachus didactylus*. Behavior. Ecol. Sociobiol. 65, 707–716.

Beçak, M.L., Beçak, W., Rabello, M.N., 1966. Cytological evidence of constant tetraploidy in the bisexual South American frog *Odontophrynus americanus*. Chromosoma 19, 188–193.

Beçak, M.L., Beçak, W., Vizotto, L.D., 1970. A diploid population of the polyploid amphibian *Odontophrynus americanus* and an artificial intraspecific triploid hybrid. Experientia 26, 545–546.

Bee, M.A., Kozich, C.E., Blackwell, K.J., Gerhardt, H.C., 2001. Individual variation in advertisement calls of territorial male green frogs, *Rana clamitans*: Implications for individual discrimination. Ethology 107, 65–84.

Bee, M.A., Cook, J.M., Love, E.K., O'Bryan, L.R., Pettitt, B.A., Schrode, K., Vélez, A., 2010. Assessing acoustic signal variability and the potential for sexual selection and social recognition in boreal chorus frogs (*Pseudacris maculata*). Ethology 116, 564–576.

Bee, M.A., 2008. Parallel female preferences for call duration in a diploid ancestor of an allotetraploid treefrog. Anim. behav. 76, 845–853.

Blumstein, D.T., Munos, O., 2005. Individual, age and sex-specific information is contained in yellow-bellied marmot alarm calls. Anim. Behav. 69, 353–361.

Brown, W.L., Wilson, E.O., 1956. Character displacement. Syst. Zool. 5, 49–64.

Brumm, H., 2006. Signalling through acoustic windows: nightingales avoid interspecific competition by short-term adjustment of song timing. J. Comp. Physiol. A 192, 1279–1285.

Bush, S.L., Gerhardt, H.C., Schul, J., 2002. Pattern recognition and call preferences in treefrogs (Anura: hylidae): a quantitative analysis using a no-choice paradigm. Anim. Behav. 63, 7–14.

Calvalcanti, M.J., 2008, Mantel for windows, Version 1.19. http://life.bio.sunysb.edu/morph/ (accessed 20.04.12).

Castellano, S., Giacoma, C., 1998. Stabilizing and directional female choice for male calls in the European green toads. Anim. Behav. 56, 275–287.

Castellano, S., Tontini, L., Giacoma, C., Lattes, A., Balletto, E., 2002. The evolution of the release and advertisement calls in green toads (*Bufo viridis complex*). Biol. J. Linn. Soc. 77, 379–391.

Castellano, S., Cuatto, B., Rinella, R., Rosso, A., Giacoma, C., 2002b. The advertisement call of the European treefrogs (*Hyla arborea*): a multilevel study of variation. Ethology 108, 75–89.

Charlton, B.D., Ellis, W.A., McKinnon, A.J., Brumm, J., Nilsson, K., Fitch, W.T., 2011. Perception of male caller identity in koalas (*Phascolarctos cinereus*): acoustic analysis and playback experiments. PLoS One 6, 1–8.

Dreher, C.E., Pröhl, H., 2014. Multiple sexual signals: calls over colors for mate attraction in an aposematic, color-diverse poison frog. Front. Ecol. Evol. 2, 22.

Duméril, A.M.C., Bibron, G., 1841. Erpétologie Genérale Ou Histoire Naturelle Complète Des Reptiles, vol. 8. Librarie Enclyclopedique de Roret, Paris.

Friedl, T.W.P., Klump, G.M., 2002. The vocal behavior of male European treefrogs (*Hyla arborea*): implications for inter- and intrasexual selection. Behaviour 139, 113–136.

Gambale, P.G., Signorelli, L., Bastos, R.P., 2014. Individual variation in the advertisement calls of a Neotropical treefrog (Scinax constrictus). Amphib. Reptil. 35, 271–281.

Gasser, H., Amézquita, A., Hödl, W., 2009. Who is calling? Intraspecific call variation in the Aromobatid frog *Allobates femoralis*. Ethology 115, 596–607.

Gerhardt, H.C., Huber, F., 2002. Acoustic Communication in Insects and Anurans: Common Problems and Diverse Solutions. University of Chicago Press, Chicago.

Gerhardt, H.C., Tanner, S.D., Corrigan, C.M., Walton, H.C., 2000. Female preference functions based on call duration in the gray treefrog (*Hyla versicolor*). Behav. Ecol. 11, 663–669.

Gerhardt, H.C., 1991. Female mate choice in treefrogs: static and dynamic acoustic criteria. Anim. Behav. 42, 615–635.

Gerhardt, H.C., 1992. Multiple messages in acoustic signals. Semin. Neurosci. 4, 391–400.

Gerhardt, H.C., 1994. Reproductive character displacement of female mate choice in the gray treefrog *Hyla chrysoscelis*. Anim. Behav. 47, 959–969.

Gerhardt, H.C., 2005. Advertisement-call preferences in diploid-tetraploid treefrogs (*Hyla chrysoscelis* and *Hyla versicolor*): implications for mate choice and the Evolution of communication systems. Evolution 59, 395–408.

Gerhardt, H.C., 2013. Geographic variation in acoustic communication: reproductive character displacement and speciation. Evol. Ecol. Res. 15, 605–632.

Grafe, T.U., 1999. A function of synchronous chorusing and a novel female preference shift in an anuran. Proc. R. Soc. Lond. B 266, 2331–2336.

Grenat, P.R., Bionda, C., Salas, N.E., Martino, A.L., 2009a. Variation in erythrocyte size between juveniles and adults of *Odontophrynus americanus*. Amphib. Reptil. 30, 141–145.

Grenat, P.R., Salas, N.E., Martino, A.L., 2009b. Erythrocyte size as diagnostic character for the identification of live cryptic *Odontophrynus americanus* and *O. cordobae* (Anura: Cycloramphidae). Zootaxa 2049, 67–68.

Grenat, P.R., Salas, N.E., Martino, A.L., 2012. Estudio de la variación morfométrica intra e interespecífica en poblaciones de *Odontophrynus* (Anura: Cycloramphidae) del área central de Argentina. Rev. Biol. Trop. 60, 1589–1601.

Grenat, P.R., Valetti, J.A., Martino, A.L., 2013. Intra-specific variation in advertisement call of *Odontophrynus cordobae* (Anura: Cycloramphidae): a multilevel and multifactor analysis. Amphib. Reptil. 34, 471–482.

Heyer, W.R., Reid, Y.R., 2003. Does advertisement call variation coincide with genetic variation in the genetically diverse frog taxon currently known as *Leptodactylus fuscus* (Amphibia: Leptodactylidae)? An. Acad. Bras. Cienc. 75, 39–54.

Higgie, M., Chenoweth, S., Blows, M.W., 2000. Natural selection and the reinforcement of mate recognition. Science 290, 519–521.

Howard, D.J., 1993. Reinforcement: origin, dynamics, and fate of an evolutionary hypothesis. In: Harrison, R.G. (Ed.), Hybrid Zones and the Evolutionary Process. Oxford University Press, Oxford, pp. 46–69.

Jang, Y., Gerhardt, H.C., 2006. Divergence in the calling songs between sympatric and allopatric populations of a wood cricket *Gryllus fultoni* (Orthoptera: Gryllidae). J. Evol. Biol. 19, 459–472.

Jouventin, P., Aubin, T., Lengagne, T., 1999. Finding a parent in a king penguin colony: the acoustic system of individual recognition. Anim. Behav. 57, 1175–1183.

Keller, M.J., Gerhardt, H.C., 2001. Polyploidy alters advertisement call structure in gray tree frogs. Proc. R. Soc. Lond. B 268, 341–345.

Lemmon, E.M., 2009. Diversification of conspecific signals in sympatry: geographic overlap drives multidimensional reproductive character displacement in frogs. Evolution 63, 1155–1170.

Loftus-Hills, J.J., Littlejohn, M.J., 1971. Pulse repetition rate as the basis for mating call discrimination by two sympatric species of Hyla. Copeia, 154–156.

Martino, A.L., Sinsch, U., 2002. Speciation by polyploidy in *Odontophrynus americanus*. J. Zool. 257, 67–81.

Otero, M.A., Grenat, P.R., Valetti, J.A., Salas, N.E., Martino, A.L., 2013. Erythrocyte nuclear size as a better diagnostic character than cell size in the identification of live cryptic polyploid species. Zootaxa 3694, 262–270.

Pettitt, B.A., Bourne, G.R., Bee, M.A., 2013. Advertisement call variation in the golden rocket frog (*Anomaloglossus beebei*): evidence for individual distinctiveness. Ethology 119, 244–256.

Pfennig, K.S., Ryan, M.J., 2006. Reproductive character displacement generates reproductive isolation among conspecific populations: an artificial neural network study. Proc. R. Soc. B 273, 1361–1368.

Pröhl, H., Koshy, R.A., Mueller, U., Rand, A.S., Ryan, M.J., 2006. Geographic variation of genetic and behavioral traits in northern and southern túngara frogs. Evolution 60, 1669–1679.

Pröhl, H., Hageman, S., Karsch, J., Hoebel, G., 2007. Geographic variation in male sexual signals in strawberry poison frogs (*Dendrobates pumilio*). Ethology 113, 825–837.

Pröhl, H., 2003. Variation in male calling behavior and relation to male mating success in the strawberry poison frog (*Dendrobates pumilio*). Ethology 109, 273–290.

Ralin, D.B., 1977. Evolutionary aspects of mating call variation in a diploid-tetraploid species complex of treefrogs (Anura). Evolution 31, 721–736.

Reers, H., Jacot, A., Forstmeier, W., 2011. Do zebra finch parents fail to recognize their own offspring? PLoS One 6, 1–7.

Schul, J., Bush, S.L., 2002. Non-parallel coevolution of sender and receiver in the acoustic communication system of treefrogs. Proc. R. Soc. Lond. B 269, 1847–1852.

Schwartz, J.J., Buchanan, B., Gerhardt, H.C., 2001. Female mate choice in the gray treefrog (*Hyla versicolor*) in three experimental environments. Behav. Ecol. Sociobiol. 49, 443–455.

Schwartz, J.J., 1987. The importance of spectral and temporal properties in species and call recognition in a neotropical treefrog with a complex vocal repertoire. Anim. Behav. 35, 340–347.

Smith, M.J., Hunter, D., 2005. Temporal and geographic variation in the advertisement call of the booroolong frog (*Litoria booroolongensis*: Anura: Hylidae). Ethology 111, 1103–1115.

Stöck, M., 1998. Mating call differences between diploid and tetraploid green toads (*Bufo viridis* complex) in Middle Asia. Amphib. Reptil. 19, 29–42.

Tarano, Z., 2001. Variation in male advertisement calls in the neotropical frog *Physalaemus enesefae*. Copeia 2001, 1064–1072.

Ueda, H., 1993. Mating calls of autotriploid and autotetraploid males in *Hyla japonica*. In: Scientific report of the Lab. Amphib. Biol. Hiroshima University, pp. 177–189.

Valetti, J.A., Salas, N.E., Martino, A.L., 2009. A new polyploid species of *Pleurodema* (Anura: Leiuperidae) from Sierra de Comechingones, Córdoba, Argentina and redescription of *Pleurodema kriegi* (Müller, 1926). Zootaxa 2073, 1–21.

Welch, A.M., Semlitsch, R.L., Gerhardt, H.C., 1998. Call duration as an indicator of genetic quality in male gray treefrogs. Science 280, 1928–1930.

Welch, A.M., 2003. Genetic benefits of a female mating preference in gray treefrogs are context-dependent. Evolution 57, 883–893.

Wong, S., Parada, H., Narins, P.M., 2009. Heterospecific acoustic interference: effects on calling in the frog *Oophaga pumilio* in Nicaragua. Biotropica 41, 74–80.