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DIVERSE TYPES OF ADVERTISEMENT CALLS IN THE FROGS *EUPSOPHUS CALCARATUS* AND *E. ROSEUS* (LEPTODACTYLIDAE): A QUANTITATIVE COMPARISON

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The variability of the advertisement calls of males from two Chilean populations of the leptodactylid frogs, *Eupsophus calcaratus* and *E. roseus* was studied and their calling behaviour further defined. Characteristic audio spectrograms and oscillograms for each species are presented. The spectral and temporal features of the calls were analysed, and intra-population and inter-specific differences in sound parameters were tested using correlation and discriminant function analysis. The calls of both species were tonal, had specific frequency modulation patterns (FM), and showed substantial inter-individual variation in several of their components. At least four discernible types of FM patterns were found in both species. Audio-spectrogram correlation and discriminant analysis showed that the quantitative characteristics of the calls of the species were clearly distinct; the most discriminating parameters were frequency, inter-call interval, and fundamental frequency.

Key words: behaviour, frequency modulation, leptodactylid, sound repertoire

INTRODUCTION

Although acoustic communication is an important feature of anuran social behaviour, each species generally has a limited repertoire of specific acoustic signals (Hauser, 1996; Bradbury & Vehrencamp, 1998). However, recent studies have revealed an important degree of plasticity in the calls of some anurans. For example in the spectral domain, male frogs may alter the dominant frequency of their calls in response to different features of acoustic stimuli (e.g., Lopez *et al.*, 1988; Wagner, 1989; Bee *et al.*, 2000; Given, 1999). In particular, an unprecedented complexity in frequency modulation (FM) has been recently reported for an Asian frog (Feng *et al.*, 2002).

The emission by males of a species-specific advertisement call and the selectivity of females for the call characteristics constitute the major pre-mating reproductive isolating mechanism in anurans (e.g. Asquith *et al.*, 1988; Gerhardt, 1974; Blair, 1958). Consequently, the study of advertisement calls is a tool that has been used extensively to elucidate taxonomic problems. Moreover, studies of mating call variation have provided useful tools for the development of evolutionary models, both at the inter-specific and inter-population level (e.g. Castellano *et al.*, 2002), and at the intra-population level in studies of communication, behaviour, and sexual selection (e.g., Friedl & Klump, 2002).

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We determined the individual and populational variation of the advertisement calls of two species of *leptodactylid* frogs from Southern Chile: *Eupsophus calcaratus* and *Eupsophus roseus*. The advertisement calls of these two species, which have similar adult body sizes and adjacent distribution ranges, have been already described in the literature (*E. calcaratus,* Formas, 1985; *E. roseus*, Formas & Vera, 1980). However, these descriptions were based on a limited number of individuals and calls: three males and 15 calls of *E. calcaratus* (Formas, 1985) and three males and an undetermined number of calls of *E. roseus* (Formas & Vera, 1980). Although these descriptions did not provide sufficient information for the quantitative comparison of the calls of *E. calcaratus* and *E. roseus,* they hinted at the complex spectral structure of the calls. Therefore, we extended those preliminary descriptions of the calls (Formas, 1985; Formas & Vera, 1980) by analysing the variation of call parameters in two geographically disjunct populations of *E. calcaratus*, and *E. roseus*. We wanted to characterise the complex FM patterns of the calls of these frogs for ancillary studies on signal recognition and speciation processes.

MATERIAL AND METHODS

Recordings of the calls of *Eupsophus calcaratus* and *E. roseus* were obtained in Chile. Recordings of *E. calcaratus* were conducted in December 1995 and October 1996 and 1997 at La Picada, Parque Nacional Vicente Pérez Rosales, Osorno Province, X región (41°6' S, 72°30' W), and recordings of *E. roseus* were done in September and October 1996 on the shores of lake Tinquilco, at the edge of Parque Nacional Huerquehue, IX región (39°47' S, 71°46' W). Recording equipment included either a Uher CR 1600 or a Sony TCD-5M tape recorder, and a Sennheiser ME 80 or an AKG CK-8 directional microphone. Substrate temperature was measured after each recording because males call from cavities in the moss that covers the soggy soil. To avoid destroying these burrow-like cavities, males were generally neither captured nor measured after recordings. The study sites were sampled on consecutive nights and cavities occupied by recorded males were marked to avoid recording from the same burrow more than once.

Recordings were processed on an Apple Macintosh computer with a digital signal analysis system. The sounds were digitised and edited at a sampling frequency of 22 kHz and 16 bit resolution with hardware and software from Sound Tools (Digidesign, USA). Canary software 1.2.4 (Cornell Laboratory of Ornithology, Ithaca, NY, USA) was used to obtain quantitative information from audio-spectrograms and oscillograms and was used to calculate audio-spectrogram batch correlations. Frequency information was obtained through fast Fourier transform (FFT, frame width 1024 points). For the calls with frequency modulation, fundamental frequency was measured at the time of peak amplitude. Frequencies of harmonics were also measured at the point of maximum amplitude. The terminology used for the description of the advertisement calls followed Heyer *et al.* (1990). The sound parameters analysed were: (1) the fundamental frequency (Fund*f*, measured in Hertz at the time of maximum amplitude of the call), (2) highest frequency of the emphasized (second and third) harmonics (H*f*2, H*f*3 measured in Hertz), (3) lowest frequency of the emphasized harmonics (L*f*2, L*f*3 measured in Hertz), (4) the relative spectral amplitude (RSA; the mean intensity of the second harmonic subtracted from the mean intensity of the third harmonic), (5) call duration (Dur; the time in seconds between the onset of a call and its end), (6) rise time proportion (RT%; the ratio between time of maximum amplitude minus onset time and call duration), and (7) the inter-call interval (ICInt; the time in seconds between the end of one call and the onset of the successive call).

We used audio-spectrogram correlations to compare the calls from both populations. From each recorded individual, we selected 800-ms recording segments with a single call; the selection was based on recording quality. Each of these selected calls was filtered to eliminate low-frequency environmental noise (high pass 250 Hz) and then normalised at 80%. The beginning and ending of each recording segment were selected so that the peak amplitude of the call occurred at 400 ms. An audio-spectrogram was calculated for each file, and all the audiospectrograms obtained were cross-correlated in a batch correlation. The process yielded a square correlation matrix containing the correlations coefficients of every call with the rest of the calls.

To determine the defining inter-specific characteristics in the calls of these two species of *Eupsophus*, a discriminant function analysis (DFA) was performed using JMP statistical package (Sall *et al.*, 2000) for Apple Macintosh computers. Functions created by DFA of the original variables maximised separation among two groups (*E. calcaratus* and *E. roseus*) and showed the contribution of sound parameters most relevant to group separation.

RESULTS

Substrate temperatures measured inside the burrow-like cavities from which frogs called varied between 8.6-10.2°C for *E. calcaratus* and between 7.8-10.5°C

TABLE 1. Mean and coefficients of variation (CV) of call parameters of *Eupsophus calcaratus* and *E. roseus*. CV_{among} is a measure of among-individual variability and it is the coefficient of variation of the mean values of the individual males. CV_{within} is a measure of the within-individual within-call period variation, calculated as the average of the individual coefficients of variation of each recording. Means are presented with their ranges and *n* is the number of individuals recorded. The suffixes 2 and 3 are second and third harmonics, respectively. Fund*f* the fundamental frequency, H*f* high frequency of the harmonic, L*f* low frequency of the harmonic, RSA relative spectral amplitude, the average intensity of the second harmonic subtracted from the average intensity of the third harmonic, Dur the duration of a call, RT% rise time proportion (the ratio between begin time subtracted from peak time and call duration), and ICInt the inter-call interval. * indicate means that were statistically different between species. * $P<0.05$; ** $P<0.01$.

		Fund*f* (Hz)	H*f*2 (Hz)	L*f*2 (Hz)	H*f*3 (Hz)	L*f*3 (Hz)	RSA (dB)	Dur (s)	RT%	ICInt (s)
E. calcaratus	mean	776.0	1486.1**	1170.0**	2157.1**	1817.2**	1.5	0.192*	0.665	5.668*
(*n*=24)	range	446.9-1103.6	1286.1-1682.8	928.7-1351.5	1804.6-2406.8	1377.3-2038.8	-9.2-10.3	0.112-0.262	0.292-0.883	0.934-16.425
	CV_{among}	33.7	5.7	9.6	6.9	9.7	72.3	22.5	25.4	79.0
	CV_{within}	9.2	3.0	2.8	2.1	3.1	21.1	12.3	25.0	44.3
E. roseus	mean	632.6	1285.2**	1037.4**	1871.0**	1618.5**	3.2	0.158*	0.609	3.417*
(*n*=18)	range	346.2-1018.6	1147.8-1428.6	875.3-1298.2	1503.1-2166.7	1194.4-1826.6	-8.0-12.5	0.124-0.235	0.271-0.844	1.479-6.947
	CV_{among}	38.7	6.1	9.5	9.1	8.7	71.7	16.7	22.0	47.0
	CV_{within}	11.6	2.9	3.0	2.8	3.3	24.4	7.19	17.9	50.5

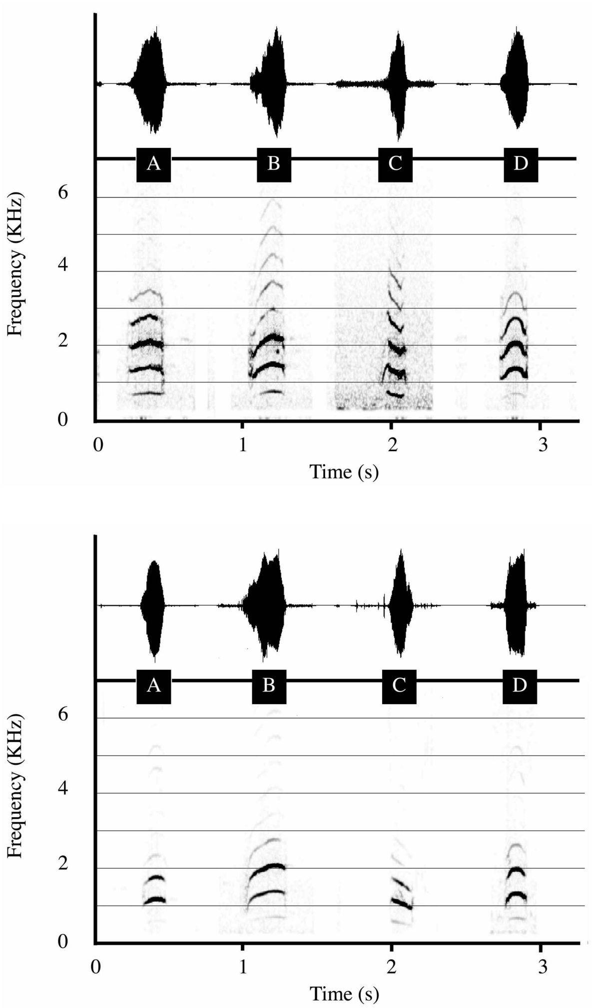

FIG. 1. Oscillogram and audio-spectrogram of the advertisement calls of four males of *Eupsophus calcaratus* and *E. roseus* with different patterns of frequency modulation (FM): A, almost flat FM; B, upwards FM; C , downwards FM; D, up-down FM. Fast Fourier Transform (FFT) details: frame width 1024 points, filter bandwidth 174 Hz, and overlap 75%.

for *E. roseus*. The advertisement calls of both species consisted of a single short FM note with a complex harmonic structure. The resulting sound resembled a short cat-like meow.

We made 30 recordings from each population. Of these recordings, a total of 263 calls from 24 individuals of *E. calcaratus* and 277 calls from 18 individuals of *E. roseus* were analysed. Some recordings included calls from more than one individual, either because they shared a portion of the burrow or because the openings of the burrows were adjacent. These recordings were excluded from the numerical analysis. However, a single call from each of these recordings was used for audio-spectrogram correlation. Table 1 summarises the quantitative parameters measured. For some calls not all sound parameters could be determined because of interference of neighbouring callers or abiotic noise. Therefore, the number of recordings for which different parameters were analysed was not the same.

In both species, each male emitted a single FM pattern within a recording session. In the calls of *E. calcaratus*, at least four basic FM patterns were clearly discernible: almost flat FM (type A, 2 of 23 males), upward FM (type B, 1 of 23 males), downward FM (type C, 4 of 23 males), and up-down (inverted U) FM (type D, 16 of 23 males); there may be additional call types with characteristics intermediate to those described here. In a given night, the call FM pattern of an individual male was largely invariable during the recording session. Advertisement call duration varied from 112-262 ms. The advertisement calls of *E. calcaratus* (Fig 1A) had a harmonic structure; the spectral energy of the first harmonic (or the fundamental) was weak. The second or the third harmonics were dominant: the second harmonic varied from 1170-1486 Hz and the third harmonic ranged from 1817-2157 Hz. In *E. calcaratus*, RSA was consistently positive in the calls of 9 males (39.13%) and the dominant harmonic was the second. Eight males (34.78%) had negative values of RSA and a dominant third harmonic, and 6 males (26.09%) had calls with positive and negative values of RSA. This value could not be measured in one individual because of excessive background noise.

In the calls of *E. roseus* (Fig. 1B), the pattern in FM was consistent within recording and at least the same four different patterns as for *E. calcaratus* were observed: almost flat FM (type A, 2 of 17 males), upward FM (type B, 1 of 17 males), downward FM (type C, 4 of 17 males), and up-down (inverted U) FM (type D, 10 of 17 males), which was again the most common case. Advertisement call duration varied from 124-235 ms. Similar to *E. calcaratus,* calls from this species had two dominant harmonics, the second (range 1037-1285 Hz) or third (1618-1871 Hz) harmonics, with the fundamental having little or no energy. Similarly to what was measured in *E. calcaratus*, in some calls of *E. roseus* the emphasised frequency was the second harmonic, whereas in other individuals it was the third. In *E.*

TABLE 2. Statistics for the sound parameters of *Eupsophus calcaratus* and *E. roseus* that were significantly different.The suffixes 2 and 3 mean second and third harmonics, respectively. H*f* high frequency of the harmonic, L*f* low frequency of the harmonic, ICInt the inter-call interval, and Dur the duration of a call. R^2_{adj} = adjusted coefficient of determination; $t_{a,b}$= Student's *t* value, a is sample size of *Eupsophus calcaratus* and b is sample size of *E. roseus*.

H*f*2	L*f*2	H*f*3	L*f*3	ICInt	Dur
R^2_{adj}=0.44	R^2_{adj}=0.26	R^2_{adj}=0.60	R^2_{adj}=0.27	R^2_{adj}=0.07	R^2_{adj}=0.16
$t_{23,17}$=5.67	$t_{23,17}$=3.84	$t_{23,18}$=7.74	$t_{23,18}$=4.03	$t_{23,18}$=2.24	$t_{24,18}$=3.20
P<0.0001	P=0.0005	P<0.0001	P=0.0003	P=0.0493	P<0.0001

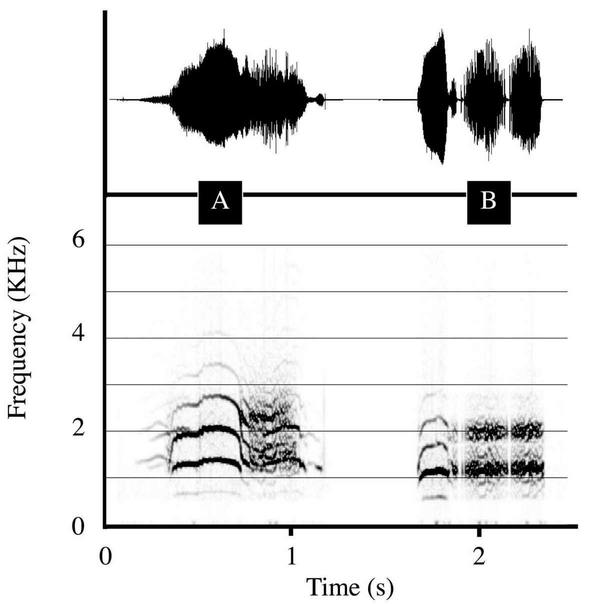

FIG. 2. Oscillogram and audiospectrogram of the aggressive calls of *Eupsophus calcaratus* (A) and *E. roseus* (B). Fast Fourier Transform (FFT) details: frame width 1024 points, filter bandwidth 174 Hz, and overlap 75%.

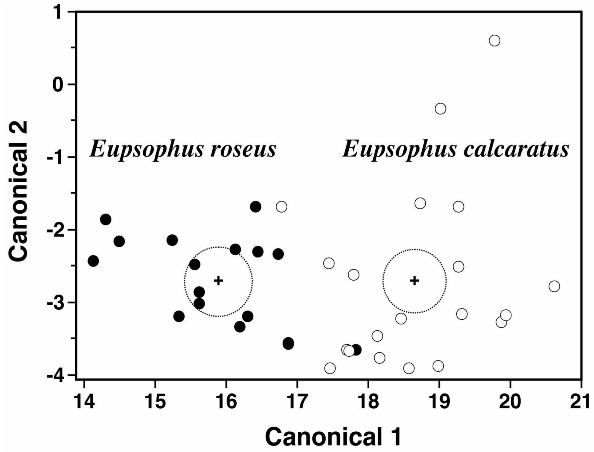

FIG. 3. Canonical functions 1 and 2 from discriminant functions analysis (DFA) performed on three sound parameters: the high-frequency of the third harmonic (H*f*3), the inter-call interval (IntCalD), and the fundamental frequency (Fund*f*), for *Eupsophus calcaratus* and *E. roseus*. The size of the dotted circle corresponds to a 95% confidence limit for the mean.

roseus, RSA was consistently positive in six males (35.29%), five males (29.41%) had consistently negative values, and six males (35.29%) showed positive and negative values. This value could not be measured in one individual because of excessive background noise.

Considering the four types of call-FM pattern, the pooled within-type mean correlations were significantly higher than the pooled among-type mean correlations for both species of *Eupsophus* that we studied (*E. calcaratus* R^2_{adj}=0.01,T=-2.24, P=0.03; *E. roseus* R^2_{adj}=0.07,T=-5.64, P<0.0001). Furthermore, the statistical comparison of individual call parameters between species showed that the frequencies of the emphasised harmonics (second and third) were all significantly different between species (Table 2), and that the duration and inter-call interval were also significantly different (Table 2). However, fundamental frequency, rise time proportion, and RSA were not significantly different between species.

In addition to the advertisement calls, a limited number of aggressive calls was recorded in both populations (three aggressive calls of one individual for *E. calcaratus*, and one aggressive call for *E. roseus*). We considered these calls to be aggressive because they were often emitted at the end of a vigorous exchange of advertisement calls among neighbouring males, or when stimulated by a loud whistle from the researchers. The oscillograms and audiospectrograms of the aggressive calls are shown in Fig 2. In the field, we observed that the aggressive calls of *E. calcaratus* promptly triggered

TABLE 3. Means (r) and standard deviations (s) of the audio-spectrogram correlations between and within populations of *Eupsophus calcaratus and E. roseus*. Self-correlations were excluded in the within population samples. *P<0.01.

	No.	r	s
E. calcaratus vs. *E. roseus*	900	0.5021*	0.08979
E. calcaratus vs. *E. calcaratus*	435	0.5788	0.07663
E. roseus vs. *E. roseus*	435	0.5675	0.10415

TABLE 4 . Summary statistics of discriminant functions and correlations between original variables and discriminant functions. * show large absolute correlations between each variable and any discriminant function. Wilks' lambda = 0.03327; Wilks' lambda exact $F_{3,33}$=22.0589, P<0.0001.

		Discriminant Functions	
		Canonical 1	Canonical 2
STATISTIC	Eigen value	2.01	-1.11×10^{-16}
	Percent	100	0
	Canonical correlation	0.8169	0
SCORING COEFFICIENTS	H*f*3	0.0113*	-0.0027
	IntCalD	0.1385	0.2941*
	Fund*f*	0.0015*	-0.0004

a neighbouring sympatric male *(Eupsophus emiliopugini)* to emit his advertisement calls. However, the number of recordings of aggressive calls is not sufficient for us to elaborate about comparative aspects.

The comparison of the calls from the two species through audio-spectrogram correlation yielded the results shown in Table 3. Given that a single call was used per male in the audio-spectrogram batch correlation, this test allowed us to use calls from all the recordings obtained (30 from each population). The average correlation was significantly higher in both within-species audio-spectrogram correlations than in the between-species audio-spectrogram correlations (*E. calcaratus* t=15.32, df=1333, P<0.0001). Furthermore, the average within-species correlation of *E. calcaratus* was not significantly higher than that of *E. roseus* (t=1.82, df=868, P=0.0687). The variation in call characteristics was greater between-species than within-species, and the within-species sample from *E. calcaratus* was slightly more homogeneous than that of *E. roseus.*

The two frog species could be discriminated by DFA based on the sound properties of their calls (Fig. 3, Table 4). As determined by stepwise DFA, the sound parameter that best discriminated the two species was H*f*3, followed by ICInt, and finally Fund*f*. Only two individuals were incorrectly classified by the test (one of each species).

DISCUSSION

The presence of four basic FM patterns in advertisement calls of *E. calcaratus* and *E. roseus* is remarkable, particularly the predominance of upward and downward FM sweep calls (type D calls). These taxa have been studied extensively from a genetic standpoint (Formas *et al.*, 1991; Formas, 1985; Formas & Brieva, 1992; Formas *et al.*, 1992; Nuñez *et al.*, 1999). It is unlikely that more than one species would be found in a single location. Therefore, the shared complex FM patterns between the species suggests a relatively recent common ancestor.

Possibly, the presence of different types of calls in these frogs is related to the relaxation of the selective pressures for call stability usually imposed by the characteristics of the acoustic environmental of anurans. In Southern Chile, the sound environment where these frogs communicate is relatively simple, and these species have few or no acoustical competitors (Penna & Veloso, 1990). *Eupsophus calcaratus* and *E. roseus* call in late winter and spring, while most other anuran species are not active. At La Picada, the only species with a calling activity period that may overlap with the calling activity of *E. calcaratus* was the congeneric species *E. emiliopugini*, and that overlap might happen only in late spring. However, the calls of *E. emiliopugini* have a substantially lower frequency (Penna & Solís, 1999; Penna & Solís, 1996). Furthermore, during this study no arthropods or nocturnal birds called nearby the breeding aggregations of these frogs.

The wide amplitude modulation range of the calls of these species of *Eupsophus* (above 1 kHz in some cases) may be related to the resonance characteristics of the burrow-like cavities in the moss from which males call (Penna, unpublished data). Given that burrows are likely to vary in shape and size, this variability in burrow filtering may also explain why small-scale features of the call may not be salient. Each cavity amplifies a specific frequency, and a considerable variation in resonant frequency occurs among cavities (842-1836 Hz for *E. calcaratus*, Penna, unpublished data). In *E. emiliopugini*, which is also fossorial and calls from burrow-like cavities, the reception of amplified conspecific calls by males inside cavities may influence the vocal interactions in choruses by amplifying calls with lower frequencies (Penna & Solís, 1996), and similar processes may also happen in the two species of *Eupsophus* that we studied. Moreover, if female frogs are in underground cavities when they first hear the advertisement calls of males, then female frogs initially will hear and potentially be attracted to male sounds amplified by both the caller's and their own cavities, and later will orient to male calls that are exclusively amplified by the caller's cavity. However, virtually nothing is known about these issues of the biology of females.

A high diversity in FM patterns has been recently reported for an Asian ranid frog, *Amolops tormotus* (Feng *et al.*, 2002). So rich is this diversity of FM patterns that no two calls were the same in over 12 hours of recordings from 21 individuals (Feng *et al.*, 2002). Although with a relatively diverse sound repertoire, the two species of *Eupsophus* that we studied are not as richly diverse as *A. tormotus*. Moreover, individuals of *A. tormotus* have a large sound repertoire, whereas the individuals of the two species of *Eupsophus* that we studied seem to emit only one call out of their species' repertoire.

Formas (1985) described the characteristics of the advertisement call of *E. calcaratus* from Puntra (Chiloé Province, Chile) and reported only one type of call with an upward and downward FM sweep, which corresponds to our type D call. The call durations measured in that description (0.15-0.21 s) are similar to the values obtained by us, whereas the reported dominant frequency range (2.2-3.8 kHz) is clearly above the frequencies measured in our study.

Formas & Vera (1980) also reported only one type of call (our type D) based on calls from three individuals of *E. roseus* from Huachocopihue, near the city of Valdivia, Chile. They reported slightly longer calls (0.19-0.2 s) and substantially higher dominant frequencies (1.6-2.9 kHz) than those measured by us in the Tinquilco population. In a more recent comparative study, an included audio-spectrogram of *E. roseus* also showed only type D calls (Formas & Brieva, 1992). Penna & Veloso (1990) also described vocalisation of *E. roseus* from Valdivia as type D calls. In relation to the harmonic structure, these authors also reported a long call similar to the aggressive call that we recorded. Other call characteristics that they reported, e.g., the second harmonic was the dominant frequency, but also recognised upper harmonics as having considerable power. The dominant second harmonic, the note durations (70-160 ms), and the dominant frequencies (0.7-1.1 kHz) are in agreement with the present observation.

In the present study, the comparative analysis of the calls of *E. calcaratus* and *E. roseus* showed an important structural similitude between the two species. The signals had at least two dominant harmonics and showed a pronounced FM in most individuals. In some cases, the range of frequency excursion was above 1 kHz. However, both the audio-spectrogram correlations and the discriminant test showed differences between the calls of the two species.

In both species, male advertisement calls showed remarkable inter-individual variation and had within individual, within-recording homogeneity. Because we recorded frogs during relatively short periods and given that most recordings were obtained from individuals that were not captured, we could not ascertain whether a male could emit more than one different call from these species' repertoires during more extended periods. However, during extended recordings of eight males of *E. calcaratus* that lasted 22-77 minutes, the individuals did not change their call type over the time sampled (Penna, unpublished data).

A further step in the study of these species could be to determine whether these frogs can discriminate among their call types, and additionally to determine which physical characteristics of the males determine the type of call produced. Furthermore, it would be interesting to determine how females perceive and respond to a given call amplified by both caller and female cavities, or exclusively by the caller's cavity. Eventually – and as has been done for *Physalaemus pustulosus* (Wilczynski *et al.*, 2001) – one could determine the extent to which the preexisting features of the receiver systems of *Eupsophus* have conditioned the evolution of their calls.

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