



Female and male phonotactic responses and the potential effect of sexual selection on the advertisement calls of a frog



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The outcome of sexual selection acting on a given sexual trait depends on the interaction between the strength, direction and form of both inter- and intrasexual selection processes. Therefore, determining female choice and male–male competition is necessary to gain a better understanding of how sexual selection acts on sexual traits. Anuran males produce advertisement vocalizations to attract females and to maintain other males at distance, providing a convenient opportunity to test the combined effect of inter- and intrasexual selection. Our main objective was to test experimentally potential effects of female choice and male–male competition and their interaction when acting as selective agents on the advertisement vocalizations of the rosy ground frog, *Eupsophus roseus*. We performed phonotaxis experiments on females and males and determined their behavioural responses to artificial signals synthesized based on the distribution of natural calls. The main results suggest that females' preference would favour vocalizations having lower frequencies and a harmonic composition in which the main harmonics of vocalizations have similar amplitudes. These preferences suggest that females could exert negative directional sexual selection on the frequency and stabilizing selection on the amplitude ratio. The responses of males were variable, suggesting that this type of male–male competition may not result in intrasexual selection favouring specific values of advertisement vocalizations. The occurrence of no clear preferences in males may result in a decrease in the potential effect of females' preferences, which could further contribute to variability in spectral components of calls.

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Female mate choice and male–male competition are well-known processes affecting the evolution of sexual traits (Andersson, 1994; Andersson & Simmons, 2006; Kokko, Jennions, & Brooks, 2006; Mead & Arnold, 2004). Inter- and intrasexual selection may act on single or correlated sexual traits and also at the same or different times. This implies the existence of complex interactions, where inter- and intrasexual selection effects can reinforce or cancel each other (Hunt, Breuker, Sadowski, & Moore, 2009; Moore & Moore, 1999). For instance, reinforcement between both types of sexual selection may arise when the same trait values that are preferred by females also increase the success in male–male competition (Berglund, Bisazza, & Pilastro, 1996; Hunt et al., 2009; Kirkpatrick & Ryan, 1991; Kokko, 2001; Mead & Arnold, 2004). On the other hand, inter- and intrasexual selection could act

differently on a given sexual trait if females prefer to mate with males that do not have the highest intrasexual competitive abilities (Arnqvist, 1992; Holland & Rice, 1998; Hunt et al., 2009; Mead & Arnold, 2004). Therefore, the combined outcome of sexual selection on a given sexual trait will depend on the interaction between the strength, direction and form of both inter- and intrasexual selection, the existence of balancing selection being a possible outcome. Determining both female choice and male–male competition and how they interact with each other is necessary to gain a better understanding of how sexual selection acts on sexual traits (Hunt et al., 2009; Moore & Moore, 1999).

Anurans and insects communicating by means of acoustic signals have been widely used as model species to study the evolution of sexual communication. This is because they use relatively simple acoustic signals that can be synthesized according to the parameters of the population distribution, a useful characteristic when exposed to controlled experimental procedures (Gerhardt & Huber, 2002; Wells & Schwartz, 2006). Males of these taxa generally produce advertisement calls, a type of signal involved in female attraction and male spacing (Gerhardt & Huber, 2002; Wells &

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Schwartz, 2006). The existence of positive phonotaxis in fertile females is well documented in a variety of species and has been commonly used as a proxy to study how intersexual selection may act on advertisement signals (e.g. Bentsen, Hunt, Jennions, & Brooks, 2006; Gerhardt, Martínez-Rivera, Schwartz, Marshall, & Murphy, 2007; Márquez & Bosch, 1997). Although males can be attracted by advertisement signals when searching for breeding sites (Lea, Dyson, & Halliday, 2002), in territorial species, maintaining other males at a distance is likely to occur, as it may increase the possibility to defend important resources for reproduction. It is important to emphasize that the characteristics of advertisement calls may have an effect on the occurrence of physical contests between callers and potential intruder males (Bee & Gerhardt, 2001; Hödl, Amézquita, & Narins, 2004; Reichert, 2010). In such cases, the dual function of advertisement signals provides the opportunity to determine how inter- and intrasexual selection interact on the same trait.

Frogs of the genus *Eupsophus* (Cycloramphidae) inhabiting the temperate forest are endemic to Chile and Argentina. Their reproductive season occurs mainly during spring. Males congregate in flooded areas where they position themselves in water-filled cavities to vocalize, actively forming dense choruses. The advertisement vocalizations produced from inside these burrows attract females and maintain separation among males (Márquez, Penna, Marques, & do Amaral, 2005; Penna & Márquez, 2007). Attracted females enter the burrows, wherein amplexus occurs; after which females leave and the males stay providing parental care to the endotherpic tadpoles (Márquez et al., 2005; Penna & Quispe, 2007; Úbeda & Nuñez, 2006). Parental care includes defending the burrows against intruder males (Márquez et al., 2005; Penna & Quispe, 2007; Úbeda & Nuñez, 2006). Although calling males established in burrows do not show phonotactic behaviour (Penna & Quispe, 2007), burrow-seeking males may enter a burrow depending on the perception and/or characteristics of calls uttered from inside these cavities. Aggressive behaviour may occur when two males encounter each other inside a burrow (Penna & Quispe, 2007). Furthermore, males with significant weight loss have been found attending the tadpoles (Úbeda & Nuñez, 2006), highlighting the importance of maintaining this resource for success in reproduction.

The males of the rosy ground frog, *Eupsophus roseus* (Duméril & Bibron, 1841) (geographical range: 38°S – 40°S), produce advertisement vocalizations consisting of a single note having a harmonic and frequency-modulated structure. The second and third harmonics present the highest energy content, and either component can correspond to the dominant frequency (i.e. the frequency component with highest energy content; Márquez et al., 2005). As burrows are an important resource for success in reproduction, we expect that females prefer advertisement vocalizations that also increase the probability of keeping burrows against potential intruding males. Therefore, we expect that inter- and intrasexual selection reinforce each other when acting on advertisement vocalizations. In the present study we tested experimentally the phonotactic behavioural response of females and males when exposed to artificial advertisement vocalizations synthesized from the distribution of natural calls. The differential response across signal values is a proxy for the mode of selection acting on sexual signals (e.g. Brooks et al., 2005; Bush, Gerhardt, & Schul, 2002; Gerhardt & Brooks, 2009). Our main objectives were (1) to establish the preference of females for specific spectral variables of advertisement vocalizations, (2) to determine which of these signal values may also attract conspecific males, and (3) to estimate how inter- and intrasexual selection interact in shaping spectral characteristics of advertisement vocalizations.

METHODS

Acoustic Recordings and Vocalization Analysis

Advertisement vocalizations of 50 *Eupsophus roseus* reproductive males were recorded in a population located within the Bosque Experimental San Martín (39°38'S, 73°07'W), a temperate forest reserve managed by the Universidad Austral de Chile. These recordings have been used in a previous study (Moreno-Gómez, Sueur, Soto-Gamboa, & Penna, 2013). A summarized description of acoustic recording and analysis is provided here. Calls were recorded using a Sennheiser ME66/K6 microphone connected to a Marantz PMD 660 digital recorder (sampling rate: 48 000 Hz, sample size: 16 bits). The distance between the microphone and the opening of the burrows from which males called was 100 cm. The call of a local male with an intercall period of 2 s was broadcasted in order to induce vocalizing animals to maintain a constant call rate. Once the focal male started to vocalize, we recorded his calls during 5 min. Recorded males were identified with a tag placed outside their burrows. Advertisement calls were recorded between September and December 2010 from 2200 to 0400 hours.

We obtained onset and offset times of vocalizations using Audacity (<http://audacity.sourceforge.net/>), and randomly selected seven clean vocalizations, allowing us to include all recorded males in the analysis. Acoustic variables of selected calls were obtained with a custom-automated analysis implemented with the R (R Development Core Team, 2013) sound analysis package 'seewave' (Sueur, Aubin, & Simonis, 2008).

To reduce computer memory consumption, audio files were down-sampled to 22 050 Hz, a sampling frequency that allows analysing the most important frequency contents of *E. roseus* calls (Márquez et al., 2005). In the middle part of calls, a fast Fourier transform was computed using a window length of 1024 points, giving a temporal resolution of 46 ms and a frequency resolution of 22 Hz. The resulting frequency spectrum was used to determine the harmonic composition measuring the frequency (Hz) and relative amplitude values in linear scale with no units (i.e. standardized between 0 and 1) of the second (F2) and third (F3) harmonics, the most important components in the advertisement vocalizations of this species (Márquez et al., 2005). The amplitudes of F2 and F3 were used to calculate the harmonic amplitude ratio (F2/F3). This variable indicates how the energy content in the two most important frequency components of advertisement calls is distributed (i.e. when the value is 1, both harmonics are equal in amplitude). This variable has no units as amplitude values were obtained in linear scale (with no units), ranging from 0 to 1. In addition, vocalization duration and the intercall period were determined. The mean for each male was calculated and then the grand mean and standard deviation were obtained. If a variable (i.e. amplitude ratio and intercall period) was skewed within an individual, prior to calculations it was log transformed to achieve normality and to calculate the mean, then the variables were reconverted to their original units. The same procedure was followed if the sampling distribution of individual means was not normally distributed. The resulting values are given in Table 1.

Acoustic Stimulus

The stimuli were synthesized using the R library 'seewave' (Sueur et al., 2008). Following the methods of Brooks et al. (2005), two types of stimuli were synthesized: (1) a standard signal that had the mean values of the acoustic variables (Fig. 1) and (2) 33 unique alternative signals. The alternative signals were synthesized using the mean vocalization duration and mean intercall period, but with varying values for F2 and amplitude ratio. The frequency of

Table 1
Characteristics of advertisement calls of *Eupsophus roseus* males ($N = 50$) and of artificial signals used in phonotaxis experiments

	Acoustic variable	Mean	SD
Natural calls	F2 (Hz)	1329	86
	AR	1.143	2.387
	DUR (s)	0.163	0.017
	ICP (s)	2.719	1.530
Artificial calls	F2 (Hz)	1335	94
	AR	1.096	2.676
	DUR (s)	0.163	—
	ICP (s)	2.719	—

F2: frequency value of the second harmonic; AR: amplitude ratio of the second and third harmonic in linear scale (no units); DUR: length of vocalizations; ICP: intercall period.

F3 was obtained using the formula $F3 = (F2/2) \times 3$, which implies a harmonic call structure (i.e. spectral peaks at integer multiples of the fundamental frequency). To obtain the values of an alternative signal, we selected two random numbers between 0 and 1 (i.e. one for each variable). We then transformed these random numbers into a z score, which represents the number of standard deviations around the mean that will take each variable. To obtain the value in the original units of the corresponding acoustic variable, we multiplied the z scores by the measured standard deviation and added the mean value of the original acoustic variables. This procedure allowed us to obtain uncorrelated synthetic signals within the original distribution of individual acoustic variables (Brooks et al., 2005). In comparison to natural signals, the use synthetic signals reduces biases in estimating selection gradients when studying potentially correlated traits (Brooks et al., 2005; Lande & Arnold, 1983). The mean and standard deviation of alternative signals is given in Table 1.

Phonotaxis Experiment

We captured females (mean \pm SD snout–vent length (SVL) = 4.1 ± 0.2 cm, $N = 33$) and males (3.7 ± 0.1 cm SVL, $N = 30$) by hand at night during the reproductive season of 2011 at sites where males were actively vocalizing and where acoustic recordings were carried out. Because it is extremely difficult to capture females in amplexus, as this activity occurs within burrows, we used the

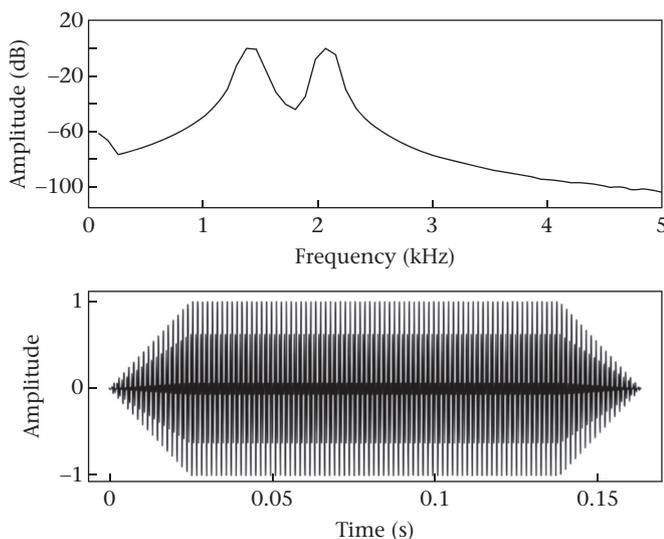


Figure 1. Artificial advertisement vocalization synthesized with the mean acoustic value of males' advertisement vocalizations. Top: mean power spectrum. Bottom: oscillogram (time versus linear relative amplitude, with no units).

presence of eggs in the female's abdomen as an indicator of female reproductive state, which was feasible because eggs were visible through the skin. Individuals were transported to the biological research station located within the Bosque Experimental San Martín, less than 1 km from the reproductive sites. The time between capture and the phonotaxis trial was no longer than 3 days. Individuals were maintained separately in plastic boxes, partially filled with water to maintain moisture. Plastic boxes were placed inside styrofoam ice chests under the forest canopy. After the experiments were carried out and prior to release, individuals were marked with Visible Implant Alpha Tags (1.2×2.7 mm, Northwest Marine Technology, Inc., Shaw Island, WA, U.S.A.) to avoid recaptures. These tags have been previously used on other anurans (Buchan, Sun, & Wagner, 2005).

Phonotaxis experiments were conducted inside a circular arena having a diameter of 70 cm and a height of 50 cm. This arena was installed in a room within the biological station. The walls of the arena were made of foam layer with 5 cm thickness to reduce acoustic reverberation. Synthetic signals were played with the Audacity software using a laptop connected to an external audio interface (Lexicon Lambda), a 30-band graphic equalizer (Suntec REQ3102) and two loudspeakers (SAMSON MediaOne 3a). The loudspeakers were placed at two extremes of the arena, facing each other. The frequency response of each loudspeaker was calibrated measuring the sound pressure level with a sound level meter (Quest Technologies Sound Pro DL 1/1) at a distance of 20 cm from the loudspeaker and controlling the graphic equalizer, resulting in a frequency response ± 1.5 dB between 0.1 and 5.0 kHz. The standard stimulus was delivered at a mean of 71.5 dB SPL root-mean square, a level within the auditory sensitivity of this species (Moreno-Gómez et al., 2013). The response of individuals to synthetic signals was recorded with a surveillance video camera with night-vision (Daiku). The camera was placed 220 cm above the centre of the arena.

At the beginning of the experiment, we placed each focal individual in the middle of the arena inside a circular, acoustically transparent bottomless cage for a 5 min period of silence. Then, we broadcast one of the 33 alternative signals. After 2.5 min, we lifted the cage and released the individual into the arena, allowing it to move freely during the next 5 min, at which time the signal broadcast ceased. If the individual did not approach the loudspeaker, we repeated the procedure but broadcast the standard signal from the opposite loudspeaker to avoid potential habituation effects. We then repeated the procedure, presenting the same alternative signal delivered at the beginning of the experiment. If an individual did not respond during the second presentation of the alternative signal, we carried out a third presentation, but switched the loudspeaker from which the alternative signal was broadcast. This procedure also helps reduce potential habituation effects of prolonged stimulation. If an individual did not approach the loudspeaker during the third presentation of the alternative signal, we suspended the experiment and did not test that individual further. For each individual, the same alternative signal was presented. Within individuals of each sex, alternative signals were not repeated. The number of presentations of standard signals varied according to the response to alternative signals. All experiments were conducted at night in complete absence of light and were performed by the same experimenter.

The videos from phonotaxis experiments were analysed with QuickTime Player 10.1. In the case of females, individuals that did not approach to within 10 cm of the loudspeaker were excluded from the analysis ($N = 2$). For the remaining 31 females, we measured each individual's latency to approach the loudspeaker. We then calculated a binary phonotaxis score by obtaining the value at which the cumulative frequency of approach time was at

50%, being 1 if females took less than 147 s and 0 if they took longer. Therefore, preferred signals were assigned a value of 1, and less preferred signals were assigned a value of 0. This binary assignment was used to comply with male phonotaxis analysis. For males, we were interested in the potential effect on the fitness of calling males; therefore, we assigned a score of 0 if a male approached to within 10 cm of the loudspeaker during signal presentation and a score of 1 if a male did not approach to within 10 cm of the loudspeaker ('nonpreference' response). We did not consider males' latency to approach the loudspeaker because not all males approached the loudspeaker, which, in contrast to female preference, does not imply that males are not responding to the stimulus.

Statistical Analysis

To determine the preference function of females and the non-preference of males for spectral variables (i.e. F2 and amplitude ratio), we used a regression approach. Because the phonotaxis score was categorized as a binary variable, a generalized linear model was fitted using R (R Development Core Team, 2013). Three models were fitted for each variable. The first model (M1) included only the intercept; therefore, it takes the assumption that the response has no variation as a function of the acoustic variable. The second model (M2) included the intercept and a linear term (β); therefore, the response is assumed to increase or decrease linearly as a function of the acoustic variable. The third model (M3) included the intercept, the linear term β and a quadratic term (γ); therefore, in this model, the response could have a concave or a convex shape as a function of the acoustic variable. The model parameter values allow estimates of the linear (β) and quadratic (γ) proxies of selection gradients and provide insight into the mode by which sexual selection acts on traits. While M1 indicates the existence of no selection, M2 indicates linear (directional) selection, and M3 indicates nonlinear (stabilizing or disruptive) selection (Brodie & Janzen, 1996; Brooks et al., 2005; Gerhardt & Brooks, 2009; Lande & Arnold, 1983; Phillips & Arnold, 1989). Prior to model fitting, we standardized predictor variables by subtracting the mean and dividing by the standard deviation. The amplitude ratio variable was log transformed, as it was not normally distributed.

These models were compared by model selection based on Akaike's Information Criteria corrected for small sample sizes with the R library 'AICcmodavg' (Mazerolle, 2013). We used this approach as it does not rely on a priori probabilities (i.e. $P < 0.05$), but instead compares the models based on their relative strength of evidence (e.g. Altwegg & Reyer, 2003). We followed the recommendations of Anderson (2008) to evaluate the strength of evidence of top-ranked models. When the top-ranked model had strong support (i.e. Akaike weight > 0.95), we obtained the parameters directly from it. If the top-ranked model had low support, we obtained parameters by shrinkage model averaging. This procedure consists in multiplying the parameter values of each model by the model weight and then summing the weighted parameter values. Finally, we estimated the combined sexual selection by summing the partial selection gradients weighted by the importance of female choice and male–male competition in determining mating success (Hunt et al., 2009). As we had no prior assessment about the importance of each of the two processes, we explored their interaction by weighting parameter values according to three scenarios: (1) higher importance of female choice relative to male–male competition (i.e. 0.75 and 0.25, respectively); (2) equal importance of female choice and male–male competition (i.e. 0.5 and 0.5, respectively); and (3) lower importance of female choice relative to male–male competition (i.e. 0.25 and 0.75, respectively). Although these values are arbitrary and other values could have been chosen, they give an approximation to different possible scenarios.

Ethical Note

All procedures were approved by the Bioethical Committee of the Universidad Austral de Chile and the Servicio Agrícola y Ganadero (SAG) from Chile (permit 4117). Individuals were treated carefully during experimentation and were released at their site of capture at the conclusion of the experiment. We were able to recapture marked frogs and they showed no signs of infection or discomfort associated with the visible implants. These implants have also been used satisfactorily in previous studies (Buchan et al., 2005).

RESULTS

The analyses of the phonotactic responses of *E. roseus* females and males when exposed to artificial advertisement vocalizations synthesized based on the variation of natural calls revealed differences between sexes: while females showed preferences, male responses were variable (i.e. no clear preferences).

In females, the model comparison of preferences for frequency values of signals (F2) indicated that the top-ranked model was the model with the assumption that responses increase or decrease linearly as a function of the acoustic variable (i.e. M2; Table 2). However, this model had a low probability, indicating that the other models could not be excluded (Anderson, 2008). The model parameters obtained by shrinkage model averaging are given in Table 3. The averaged model indicated that females preferred signals that were below the mean of the distribution (i.e. signals lower in frequency), suggesting that females could exert negative directional selection on this variable of advertisement vocalizations (Fig. 2c). For the amplitude ratio of the second and third harmonic (amplitude ratio), the top-ranked model had a high probability (Table 2). This model corresponds to M3, which takes the assumption of a concave or convex preference. In this case, females preferred signals having amplitude ratio values around the mean of the distribution (Fig. 2d). This suggests that females may exert stabilizing selection on amplitude ratio. Female preference for these values suggests that signals characterized by similar amplitudes of F2 and F3 could be favoured by intersexual selection (Tables 2, 3, Fig. 2c, d).

In the case of males, we did not find any clear differential response across the values of F2. The model parameters obtained by shrinkage model averaging are given in Table 3. The results suggest that low-frequency signals are slightly less attractive to potential intruding males (Fig. 2e). In the case of amplitude ratio, a similar tendency was observed (Fig. 2f). The resulting average model suggested that signals with amplitude ratios characterized by higher amplitudes in F2 than in F3 could be slightly less attractive to potential intruding males, but nevertheless, as with F2, this result should be interpreted cautiously.

When estimating the interaction between inter- and intrasexual selection, the analysis indicated that the occurrence of no clear responses in males resulted in a decrease in the potential effect of females' preference, where the magnitude of this decrease depended on the relative weight used on each scenario. In spite of this, the tendencies remained similar to the ones observed in females' preferences: there was negative directional sexual selection on F2 and stabilizing sexual selection on amplitude ratio (Fig. 2g, h, Table 4).

DISCUSSION

The analyses of the phonotactic responses of *E. roseus* females and males exposed to artificial advertisement calls indicate differences between the sexes. While in the case of females we found preferences in both tested variables (i.e. F2 and amplitude ratio), in

Table 2
Model selection of responses by female and male *Eupsophus roseus* to spectral variables of male advertisement calls

Sex	Variable	Model	K	AICc	ΔAICc	AICc wt	LL	β	γ
Female	F2	M2	2	43.799	0.000	0.549	−19.685	−0.742 (0.421)	—
		M1	1	45.081	1.282	0.289	−21.471	—	—
		M3	3	46.239	2.440	0.162	−19.675	−0.752 (0.430)	−0.053 (0.371)
	AR	M3	3	35.653	0.000	0.984	−14.382	1.046 (0.697)	−1.756 (0.691)
		M1	1	45.081	9.428	0.009	−21.471	—	—
		M2	2	45.504	9.851	0.007	−20.538	0.516 (0.390)	—
Male	F2	M1	1	43.732	0.000	0.484	−20.794	—	—
		M2	2	44.367	0.635	0.352	−19.961	−0.495 (0.396)	—
		M3	3	45.901	2.170	0.164	−19.489	−0.535 (0.422)	−0.364 (0.388)
	AR	M1	1	43.732	0.000	0.573	−20.794	—	—
		M2	2	45.590	1.858	0.226	−20.573	0.250 (0.378)	—
		M3	3	45.835	2.103	0.200	−19.456	0.282 (0.412)	0.522 (0.367)

Model selection based on Akaike's Information Criteria corrected for small sample sizes (AICc). F2: frequency of the second harmonic; AR: amplitude ratio of the second and third harmonic in logarithmic scale (no units); K: number of estimated parameters for each model; ΔAICc: difference between the best model and each of the other models. AICc wt: weight of evidence of the model; LL: log likelihood of each model; M1 indicates the existence of no selection; M2 indicates linear selection (directional); M3 indicates nonlinear selection (stabilizing or disruptive). Models are ordered by rank, from highest to lowest. Estimates and SEs are given for linear (β) and quadratic (γ) terms of each model (when corresponding).

the case of males there were no clear responses for any specific signal value.

In females, we found a preference for lower-frequency signals, which suggests the potential existence of negative directional selection on this variable of advertisement vocalizations. This result should be taken with caution, however, because of the relatively low support for the top-ranked model. In spite of this limitation, model averaging yielded the same trend. Although this result is in agreement with the expectation that anuran females prefer lower-frequency calls as they could signal large male body size (Gerhardt & Huber, 2002), in *Eupsophus* frogs this relationship is not clear (Penna, 2004). A potential explanation for the females' preference for low-frequency signals could be related to the characteristics of their auditory sensitivity (Ryan & Keddy-Hector, 1992; Ryan, Perril, & Wilczynski, 1992; Gerhardt & Schwartz, 2001). *Eupsophus roseus* females have greater auditory sensitivity for the third harmonic of advertisement vocalizations, specifically between 1700 and 2100 Hz (Moreno-Gómez et al., 2013). Because harmonics are integer multiples of the fundamental frequency, the second harmonic should be between 1130 and 1400 Hz, values that are between the lower range and the mean of the distribution of advertisement vocalizations (Table 1, Fig. 2a). In the case of the amplitude ratio between the second and third harmonics, the results suggest that females prefer values around the mean of the distribution, suggesting that females may exert stabilizing selection. The preferred values correspond to advertisement vocalizations having similar amplitude in the second and third harmonics, which could elicit a higher stimulation on females' auditory sensitivity (Moreno-Gómez et al., 2013). Previous studies on female anuran preferences indicate that females may exert weak

directional or stabilizing selection on spectral variables (e.g. Gerhardt, 2005; Gerhardt & Brooks, 2009). Such tendency is expected to relate to the matching between the spectral characteristics of advertisement vocalizations and the auditory sensitivity of females, which enhances the signal-to noise ratio (Gerhardt & Brooks, 2009; Gerhardt & Schwartz, 2001; Moreno-Gómez et al., 2013; Ryan & Keddy-Hector, 1992; Ryan et al., 1992; Wilczynski, Rand, & Ryan, 2001).

In males, we did not find any clear differential response across the values of the variables tested, but the strength of evidence for the top-ranked model for each variable was low; therefore, the possibility of a clear response cannot be excluded (Anderson, 2008). It is possible that the sample we used for estimating males' responses included males with different competitive abilities, and thus, males that could potentially react in different ways to advertisement signals (e.g. Humfeld, 2008; Jang, 2011; Leonard & Hedrick, 2009). Two competing hypothesis are the 'satellite male hypothesis', which proposes that unattractive males congregate near attractive males in an attempt to intercept females, and the 'aggressive displacement hypothesis', which proposes that males congregate to engage in calling contests. Further studies should evaluate these hypotheses by associating the attractiveness, fighting abilities and body sizes of males with the calls that are most preferred by females (e.g. Leonard & Hedrick, 2009).

Although in other anurans vocal interactions between calling males could result in intrasexual selection (Wells & Schwartz, 2006), in the case of the congeneric species *Eupsophus calcaratus*, males do not respond differentially to calls differing in spectral characteristics (Penna & Quispe, 2007). This may suggest that in *E. roseus* this form of male–male competition could not have a clear effect on the spectral characteristics of advertisement vocalizations.

When considering the three potential scenarios of the interaction between inter- and intrasexual selection, the results still suggest the existence of negative directional selection on the frequency and stabilizing selection on the amplitude ratio. This suggests that female choice is of greater importance than male–male competition in shaping the distribution of acoustic variables. These results are in agreement with previous studies (e.g. Sullivan-Beckers & Coccoft, 2010), however, they also suggest that male–male competition could help to maintain the variability of spectral variables of advertisement vocalizations. It is important to consider that other acoustic variables (i.e. temporal variables) could affect the selective process, as in anurans and insects there is evidence that both temporal and spectral variables are targets of sexual

Table 3
Estimated parameter values based on Akaike's model selection and shrinkage parameter averaging

Sex	Acoustic variable	Intercept	β	γ
Female	F2	−0.060	−0.529	−0.009
	AR ^a	1.230	1.046	−1.756
Male	F2	0.052	−0.262	−0.060
	AR	−0.096	0.113	0.104

F2: frequency value of the second harmonic; AR: amplitude ratio of the second and third harmonic in linear scale (no units).

^a In the case of female–AR, parameter values were obtained directly from the best model.

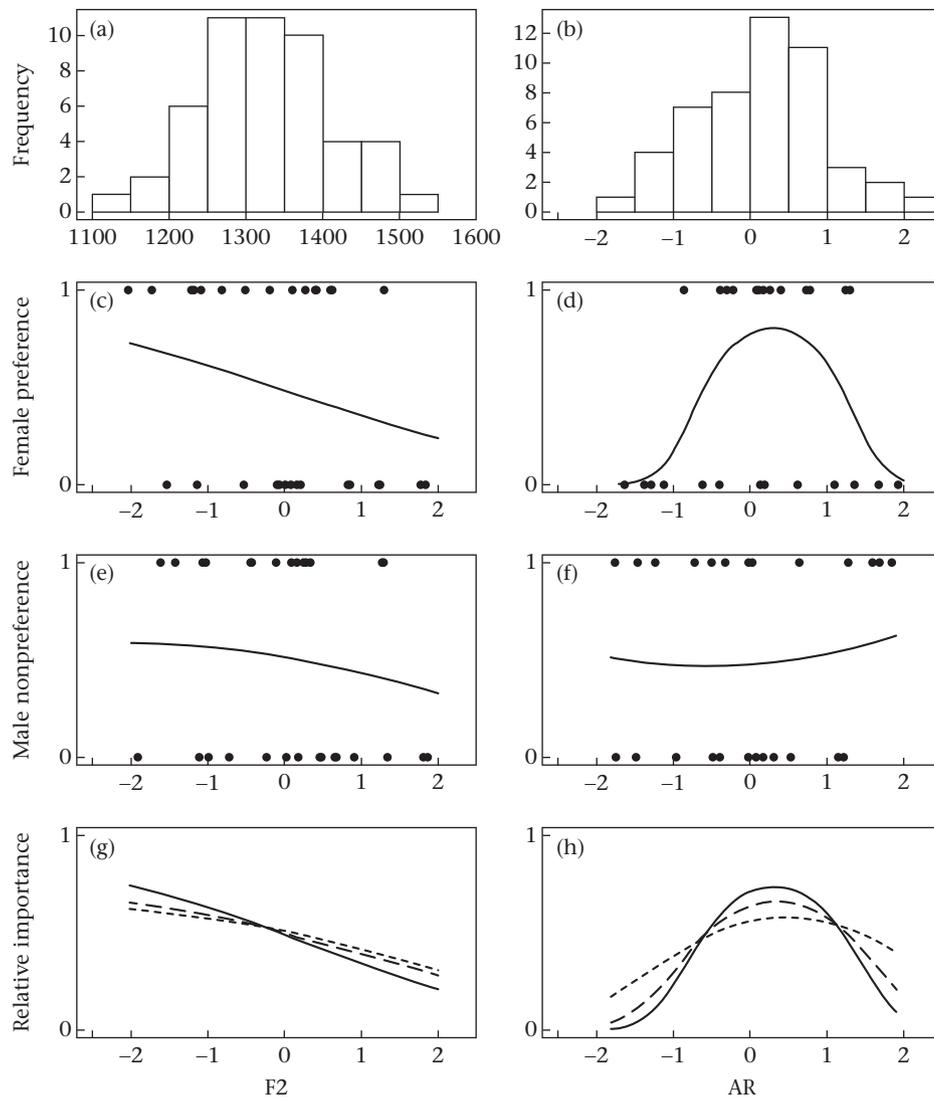


Figure 2. Distribution of acoustic variables of advertisement vocalizations produced by *Eupsophus roseus* males ($N = 50$): (a) frequency (Hz) of the second harmonic (F2); (b) amplitude ratio (AR) between the second and third harmonic in logarithmic scale (with no units). Female ($N = 31$) preference and male ($N = 30$) nonpreference for the tested acoustic variables: (c) preference of females for F2; (d) preference of females for AR; (e) nonpreference of males for F2; (f) nonpreference of males for AR; curves correspond to the predicted model obtained by Akaike's model selection. Relative importance of inter- and intrasexual selection for (g) F2 and (h) AR under three scenarios: intersexual selection > intrasexual selection (solid line); intersexual selection = intrasexual selection (dashed line) intersexual selection < intrasexual selection (dotted line). Variables are standardized in panels (c) – (h) (see [Methods](#) for details).

Table 4

Estimated combined inter- and intrasexual selection parameters for the tested spectral variables of *E. roseus* advertisement vocalizations under three scenarios differing in the importance of each form of selection^a

Acoustic variable	Parameter	Inter>Intra	Inter=Intra	Inter<Intra
F2	Intercept	-0.032	-0.004	0.024
	β	-0.593	-0.396	-0.329
	γ	-0.021	-0.034	-0.047
AR	Intercept	0.899	0.567	0.236
	β	0.813	0.580	0.346
	γ	-1.291	-0.826	-0.361

F2: frequency value of the second harmonic; AR: amplitude ratio of the second and third harmonic in linear scale (no units).

^a Original parameters were obtained from Akaike's model selection and shrinkage averaging.

selection (e.g. [Bentsen et al., 2006](#); [Brooks et al., 2005](#); [Gerhardt & Brooks, 2009](#); [Sullivan-Beckers & Cocroft, 2010](#)). Furthermore, our laboratory results need to be corroborated by studies of male mating success in the field (e.g. [Bentsen et al., 2006](#); [Friedl, 2006](#); [Sullivan-Beckers & Cocroft, 2010](#)).

A potential explanation for the lack of equilibrium between females' preferences and the mean population call frequency value could be related to masking effects of background noise, mainly from calls of the congeneric and syntopic species *Eupsophus vertebralis* ([Moreno-Gómez et al., 2013](#)). This could imply an interaction between natural and intersexual selection when favouring different values across the call frequency distribution. Advertisement calls could also have a low heritable variation, implying that these signals may not respond rapidly to selection ([Arnold & Wade, 1984a, 1984b](#)). Although there are reports of significant heritability estimates in acoustic sexual signals, the opposite has also been found ([Brandt & Greenfield, 2004](#); [Butlin & Hewitt, 1986](#);

Forstmeier, Burger, Temnow, & Derégnaucourt, 2009; Ritschard & Brumm, 2011; Simmons, Tinghitella, & Zuk, 2010; Webb & Roff, 1992; Welch, Smith, & Gerhardt, 2014). Furthermore, the frequency of signals can be genetically correlated with other components of acoustic signals, decreasing the potential of achieving equilibrium between preferences and this variable of advertisement calls (Forstmeier et al., 2009; Welch et al., 2014). Moreover, female preferences may fluctuate among reproductive seasons, meaning that intersexual selection would favour calls with different frequency values at different breeding times (Coleman, Patricelli, & Borgia, 2004; Jennions & Petrie, 1997; Svensson & Gosden, 2007).

Understanding the factors involved in the evolution of acoustic sexual signals is important to elucidate causes of population divergence and, therefore, those potentially associated with speciation processes (Wilkins, Seddon, & Safran, 2013). Future studies should evaluate the effects of sexual selection on different populations of *E. roseus*. This would contribute to our understanding of the relative importance of inter- and intrasexual selection on signal population divergence, as contrasting results have been reported across species (e.g. Bush & Schul, 2010; Claridge & Morgan, 1993; Lemmon, 2009; Rodríguez, Ramaswamy, & Crocroft, 2006; Velásquez, Opazo, Díaz, & Penna, 2014).

Overall, we found that female preferences may exert negative directional selection on call frequency and stabilizing selection on the amplitude ratio between the second and third harmonics. In addition, we found that males showed no clear differential responses across the values of spectral variables examined. When considering the interaction between both proxies of intra- and intersexual selection, we found that the variable response of males generated a decrease in the potential effect of females' preference on the spectral variables of advertisement vocalizations. These results highlight the importance of determining both forms of sexual selection. However, future studies should evaluate the mating success of *E. roseus* in the field and also determine whether male advertisement calls show heritable variation, which is needed for the evolutionary response to selection.

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