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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.

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 endothrophic 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