

1 **Call overlapping signals sexual status in Darwin's frogs**

2 José M. Serrano^{1,2}, Noé Guzmán³, Mario Penna¹, Marco A. Méndez⁴, Claudio Soto-Azat⁵

3
4 ¹ Programa de Fisiología y Biofísica, Facultad de Medicina, Universidad de Chile, Santiago, Chile

5
6 ² Laboratorio de Comunicación Animal, Facultad de Ciencias Básicas, Universidad Católica del Maule, Talca, Chile.

7
8 ³ Laboratorio de Fisiología Animal, Facultad de Ciencias, Universidad de Chile, Santiago, Chile

9 ⁴ Laboratorio de Genética y Evolución, Facultad de Ciencias, Universidad de Chile, Santiago, Chile

10
11 ⁵ Sustainability Research Centre, Life Sciences Faculty, Universidad Andres Bello, Santiago, Chile

12
13
14 Corresponding Author: José M. Serrano

15
16 Email address: jserrano@ucm.cl

Abstract

Background. In animal reproductive contexts, calling behaviour is mostly performed by males but in species in which females call, it is not known how vocal interaction occurs between sexes, particularly when sexual dimorphism in signals is low, as in cases in which call repertoire is identical but acoustic properties differ. In Darwin's frog (*Rhinoderma darwinii*), a species in which males brood larvae inside their vocal sacs, females have higher dominant frequency and shorter calls and notes than males. Since in this species males persist calling after getting pregnant with larvae, different vocal interaction patterns are expected to occur among animals having dissimilar reproductive status.

Methodology. To explore the mechanisms underlying vocal recognition among the different sexual status of *R. darwinii*, we recorded natural duets between non-pregnant males (NPM), pregnant males (PM) and females (F) and evaluated their evoked vocal response to natural playback stimuli of each sexual status from November to February 2015-2016 in Chiloé island, Chile. Call rate, phase angles, sound pressure level (SPL), number of overlapping calls and delay of overlapping calls were measured to determine differential responses between natural duets and in response to stimuli consisting of natural calls of individuals of different sexual status.

Results. Spontaneous duet interactions occurred mainly between males and no clear differences between duets were detected. In playbacks, call ratios in response to calls of different sexual status were similar. Females decreased their SPL in response to F calls, while F and PM had longer call delays and lower call overlaps between each other. Major differences were observed in call overlap, as the occurrence of this phenomenon was larger in playback experiments than during natural duets. The number of calls overlapped during natural duets was fewer (10.9 %) than during playback experiments (36.8 %).

Conclusions. Our results suggest that in *R. darwinii*, PM and F signalize their sexual status by decreasing their call overlap and that NPM respond indistinctly to the other sexual status. In general, these differences in selective call overlap between Darwin's frogs arise as a novel mechanism for signal recognition between animal vocal interactions.

Introduction

The display of sexual signals has been mostly considered an exclusive feature of males (Price 2015), however, growing evidence has shown that females can display sexual signals in various taxa (e.g. Serrano and Penna 2018), questioning their exclusive role as mediators of female choice and competition between males (e.g. Tobias, Montgomerie and Lyon 2012). In addition, the study of duets and choruses formed by males and females may contribute new explanations about the role of signal exchanges in social and sexual processes (e.g. Janik and Slater 1998; Cui et al. 2010; Fishbein et al. 2018). In this regard, a largely unexplored issue in animal communication is how the timing of acoustic signals involved in recognition of conspecifics contribute to group cohesion in complex societies (Sheehan and Bergman 2016).

Darwin's male frogs brood in their vocal sac larvae collected from eggs laid by females and fertilized by males (Goicoechea Garrido and Jorquera 1986). In the field, Darwin's frogs

usually call isolated, in pairs or in small groups on moss mounds on undergrowth in temperate forest environments (Crump 2002). However, the occurrence of sexual and social interactions within and between sexes in Darwin's frogs have not yet been determined. Recently advertisement call of this species has been shown to possess a sexual dimorphism related to body size differences between males and females but lacks clear differentiation between males with different pregnancy status (Serrano 2019). The aim of the current study is to understand the role of vocal signalling for sexual recognition in a social environment conformed by males and females. It also expects shed light to understand the role of vocal interaction in a social environment conformed by male individuals with distinct sexual status. In this study we evaluate the hypothesis that Darwin's frogs recognize their sexual identity by means of their calls, by recording natural vocal interactions between individuals of different sexual status and conducting evoked vocal response (EVR) experiments with stimuli representing the diverse sexual status.

Materials & Methods

We describe patterns of vocal interactions in a social environment conformed by pregnant males (PM), non-pregnant males (NPM) and females (F), recording natural duets between animals having these three status during the reproductive season lasting five months (October 2015 to February 2016) in a population located on the Island of Chiloé, Chile (43° 21' S; 74° 6' W). In addition, we evaluated EVR to playbacks of natural calls of individuals of the three sexual status.

Duet recordings

Vocalizations of subjects calling in duets were recorded with a digital recorder (Tascam DR-100) at a sampling rate of 44.1 kHz and 16-bit resolution and two directional microphones (Sennheiser ME-66) plugged to each recording channel. The distance separating the two subjects intervening in the duet was measured and sound pressure level (SPL re 20 µPa, C frequency weighting and fast time weighting) of calls of one individual conforming the duet was recorded placing a sound level meter microphone (Extech 407780) adjacent to the tip of the directional microphone. Latency (registered as phase angle of call onsets between the calls of the two individuals; Klump and Gerhardt 1992), number of call overlaps and delay between the onset of overlapping calls between interacting subjects were measured. To discard that call overlap was occurring by chance between pairs of individuals composing a duet, number of overlaps and overlap delay between duets was compared using generalized linear models (GLM).

Playback experiments

Call bouts of playback stimuli were composed of 10 natural calls of individuals of the three sexual status having a high signal to noise ratio. The amplitude of call bouts was standardized at 64 dB SPL at the position of the subjects and time intervals between successive calls within a call bout were generated with random intervals of silence lasting 5 – 60 s. These values approximate those occurring in natural interactions between individuals of Darwin's frog. Following this procedure, bouts of calls having different call rates and lasting 138 – 399 s resulted. This randomization in call timing allowed to evaluate the temporal relationship of the EVR to the stimuli, independent of potential rhythmic calling behaviour based on an internal oscillator (Zelick

and Narins 1985). Three-minute silent intervals spaced call bouts of the different stimuli and the order of presentation of call bouts of each sexual status was randomized. Stimuli were presented with a Samsung J1 WAV player connected via Bluetooth to a portable loudspeaker (i.Sound 5464). Spontaneous vocal activity of the experimental subjects was recorded and thereafter playbacks of calls of the three sexual status were presented sequentially through a loudspeaker placed on moss vegetation at 1 m and at an angle of about 90 degrees relative to the focal subject. Upon completion of each playback experiment, identity of focal individuals was registered. Responses to natural stimuli were analyzed measuring call rate, latency, SPL and number of overlaps of response calls with the stimuli as for duets. Call rate and SPL were computed for periods of silence and stimuli presentation, while latency and number of overlaps were measured only for stimuli presentations. GLM, ANOVA and post-hoc tests were used to compare responses to stimuli of the three sexual status.

Results and Discussion

Duet recordings

Thirteen interactions were recorded between individuals belonging to the three sexual status: between NPM (N= 5), PM (N= 3), NPM and F (N = 4), and NPM and PM (N= 1). SPLs of the calls were not affected by the distance from the focal individual at which this variable was measured (range= 18 to 68 cm), as no significant correlation between this amplitude measure and recording distance occurred ($n= 18$; $r= -0.13$; $df= 16$; $p= 0.612$). No clear pattern was observed between the different kinds of duets regarding call rates, phase angles and SPL. Ratios between overlapping relative to non-overlapping calls were different from chance in duets composed by NPM, by NPM and F, and by PM (Table 1). As NPM was the only sexual status observed interacting with the two other sexual status, its responses to the three sexual status were compared, showing that calls of this sexual status overlapped at an earlier time with F than with both types of males (ANOVA test, $\chi^2= 6.972$; $p < 0.05$; Fig. 1).

Table 1. Percentage of overlapping calls in vocal interactions between non-pregnant males (NPM), pregnant males (PM) and females (F) of Darwin's frog in natural duets and with calls of these three sexual status in playback experiments. Significant differences between the numbers of overlapping and non-overlapping calls: $*= p < 0.05$; $**= p < 0.01$; nr= duets not recorded.

Interaction	% of overlapping calls between sexual status								
Focal individual	NPM	NP M	NPM	PM	PM	PM	F	F	F
Preceding caller or stimuli	NPM	PM	F	NPM	PM	F	NPM	PM	F
Natural duets	11.4**	16.3	12.3**	3.7*	10.6**	nr	22	0	nr
Playback experiments	29.8	48.8	42.1	42.1	36.4	5*	53.3	39.5	33.9

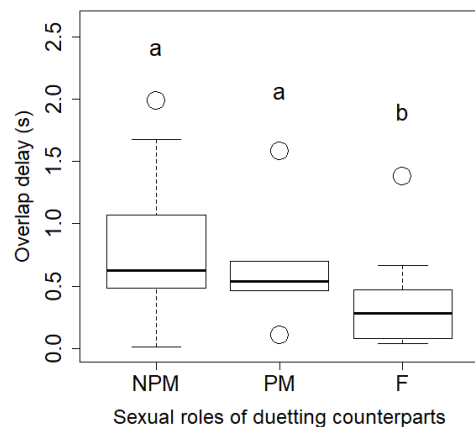


Fig. 1. Delays between the onsets of overlapping calls for duets between non-pregnant males in response to non-pregnant males (NPM), pregnant males (PM) and females (F). Different low-case letters (a, b) indicate significant differences in post-hoc analyses (Tukey tests, $p < 0.05$).

Playback experiments

Thirty-two individuals were stimulated with natural calls, 14 of which were NPM, 12 PM and six F. Call ratios in response to calls of different sexes were similar. However, F decreased their SPL in response to F calls relative to the initial silent period (GLM test, $t=3.136$; $p<0.01$; Fig. 2A) and had longer latency to PM relative F and NPM stimuli (GLM test, $t= -2.573$; $p< 0.05$; Fig. 2B), while PM had lower number of overlapping calls to F relative to NPM and PM (GLM test, $z= -1.691$; $p<0.05$; Fig. 2C).

The occurrence of call overlaps was larger in playback experiments relative to duet interactions (GLM test, $z= 8.11$; $p< 0.001$; Table 1) and overlap delay in response to all stimuli differed between sexual status, as F responded with a shorter overlap delay to all the stimuli combined relative to both types of males (ANOVA test, $\text{Chi}^2= 7.107$; $p< 0.05$; Fig. 3). Such short overlap delay of F during playback contrasts with the lower overlap delay observed also in F duetings (Fig. 1; see in Table 1 that F were observed overlapping calls with NPM only).

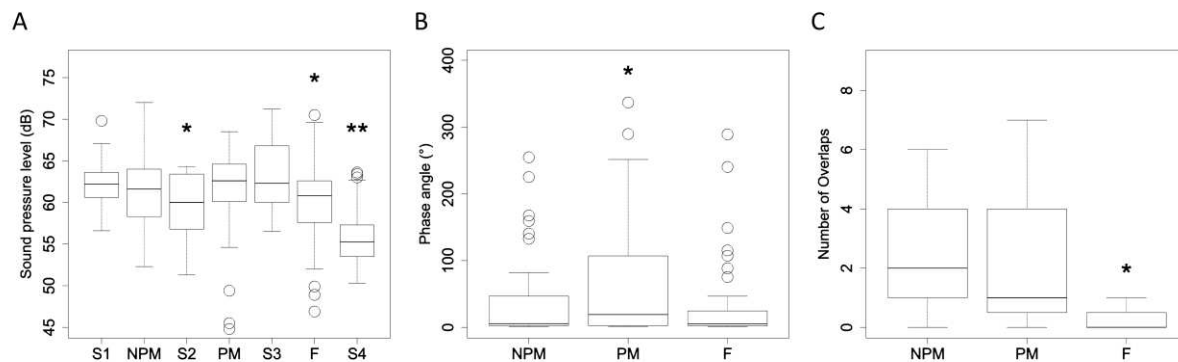


Fig. 2. Sound pressure level (A) and phase angle (B) in evoked calls of females, and number of overlaps (C) in evoked calls of pregnant males in response to natural stimuli of the three sexual status. Stimuli abbreviations: NPM: non-pregnant males, PM: pregnant males, F: females. S1, S2, S3 and S4: silent intervals between stimuli presentations. Asterisks indicate significant differences in post-hoc analyses relative to S1 in A, and between stimuli in B and C (Tukey tests, $*=p<0.05$, $**=p<0.01$)

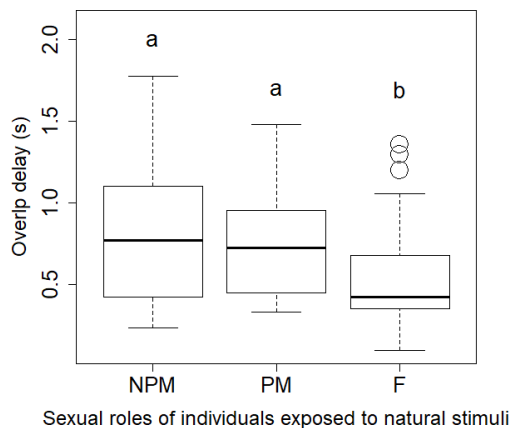


Fig. 3. Overlap delays observed for non-pregnant males (NPM), pregnant males (PM) and females (F) in response to playbacks of all natural stimuli combined. Different low-case letters (a, b) indicate significant differences in post-hoc analyses (Tukey test, $p < 0.05$).

Our results suggest that in Darwin's frogs signal recognition is not evinced in gross measures of vocal activity such as call rate like it occurs in other species (e.g. Cui et al. 2010; Fishbein et al. 2018). However, subtle differences in call overlap apparently indicate dissimilar readiness to interact vocally between individuals of different sexual status. PM and F of *R. darwinii* are relatively selective in their modes of synchronization with calls of different sexual status and NPM interact similarly with all the sexual status, a strategy likely to favour spatial tolerance of potential breeding partners. These differences in selectivity in call overlap between duetting pairs may contribute a novel mechanism of sexual recognition that could be relevant for acoustic interactions among other organisms and artificial devices.

Acknowledgements

Mara Santoyo, Nicolette Thompson, Matías Muñoz, María Luisa Estay and Jaime Beltrand helped in obtaining field data.

References

Cui, J., Wang, Y., Brauth, S. and Tang, Y. (2010). A novel female call incites male-female interaction and male-male competition in the Emei music frog, *Babina daunchina*. *Animal Behaviour*, 80: 181–187.

- Crump, M. L. (2002). Natural history of Darwin's frog, *Rhinoderma darwinii*. Herpetological Natural History 9: 21–30.
- Fishbein, A. R., Löschner, J., Mallon, J. M. and Wilkinson, G. S. (2018). Dynamic sex-specific responses to synthetic songs in a duetting suboscine passerine. PLoS ONE 13: e0202353.
- Goicoechea, O., Garrido, O. and Jorquera, B. (1986). Evidence for a trophic paternal-larval relationship in the frog *Rhinoderma darwinii*. Journal of Herpetology, 20:168–178.
- Janik, V. M. and Slater, P. J. (1998). Context-specific use suggests that bottlenose dolphin signature whistles are cohesion calls. Animal Behaviour, 56: 829-838.
- Klump, G. M. and Gerhardt, H. C. (1992). Mechanisms and function of call-timing in male-male interactions in frogs. In: McGregor, P. K. (ed). Playback and studies of animal communication. New York, USA: Springer Science & Business Media, p. 153-174.
- Price, J. J. (2015). Rethinking our assumptions about the evolution of bird song and other sexually dimorphic signals. Frontiers in Ecology and Evolution, 3: 40.
- Serrano, J. M. (2019). El rol de las señales acústicas en las interacciones sexuales y la estructura social de la ranita de Darwin (*Rhinoderma darwinii*). Doctoral Thesis, Universidad de Chile.
- Serrano, J. M. and Penna, M. (2018). Sexual monomorphism in the advertisement calls of a Neotropical frog. Biological Journal of the Linnean Society, 123: 388–401.
- Sheehan, M. J. and Bergman, T. J. 2016. Is there an evolutionary trade-off between quality signaling and social recognition? Behavioral Ecology 27: 2–13.
- Tobias, J. A., Montgomerie, R. and Lyon, B. E. (2012). The evolution of female ornaments and weaponry: social selection, sexual selection and ecological competition. Philosophical Transactions of the Royal Society B, 367: 2274–2293.
- Zelick, R. and Narins, P. M. (1985). Characterization of the advertisement call oscillator in the frog *Eleutherodactylus coqui*. Journal of Comparative Physiology A, 156: 223–229.