



Monomorphic call structure and dimorphic vocal phenology in a sex-role reversed frog

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Abstract

Sexual signals in different animals are expected to be dimorphic when both sexes signal, but cases of monomorphism are known to occur, and we lack a clear understanding about the factors that modulate the level of sexual dimorphism in signals. In this study, we evaluated the hypothesis that the lack of dimorphism in sexual signals might evolve in systems experiencing temporal changing conditions of intra-sexual competition. We used the Darwin's frog (*Rhinoderma darwinii*), a species with paternal care, as a model. We compared advertisement calls and examined call distinctiveness among females, pregnant and non-pregnant males in a wild population from Chiloé island, Chile. We also recorded the vocal activity of both sexes along the reproductive season. Additionally, we compared the acoustic properties of their advertisement calls in terms of sexual distinctiveness and individual repeatability. We found that the proportion of females and pregnant males vocalizing changed over time following distinct patterns. Females produced calls with lower dominant frequency and longer note and call durations than males, and these acoustic differences were related to body size differences between sexes, but only dominant frequency contributed significantly to the distinctiveness of calls between sexes. Also, individual repeatability was high, indicating that calling can be relevant for social recognition. Overall, our results suggest that mutual selective pressures could be involved in the limited dimorphism of the advertisement calls in Darwin's frogs, as the sex ratio of individuals vocalizing (i.e. females vs. reproductive males) is reversed along the breeding period.

Significance statement

Whether sexually monomorphic signals are evidence of adaptive mutual choice or a by-product of genetic constraints on females remains as an open question. In species with exclusive parental care of males, it would be expected that males and females alternate their reproductive availability while performing slightly differentiated sexual signals. Using acoustic recordings and capture-recapture data of the Darwin's frog, we found that advertisement calls of this frog tend to be monomorphic. Interestingly, the males performing parental care were calling actively and the population had a clear bias in the number of males. Males and females of this endangered frog called actively, but the vocalization rate of each sex peaked at different times along the breeding season. These findings open new questions about the mechanisms of sexual recognition under restricted signal dimorphism.

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Introduction

Signals displayed by animals provide key sexual and social information (e.g. Laidre and Johnstone 2013). Displays related to reproduction can be produced by males, females or both sexes (e.g. Colley and Parmentier 2012; Riebel et al. 2019). In species where males produce signals, they usually produce exaggerated displays to attract females in competitive contexts (e.g. Ryan 1998), but these signals can in addition mediate individual and social recognition (e.g. Bee 2016; Sheehan and Bergman 2016). In species in which both sexes signal, displays of males and females may also mediate diverse recognition processes (e.g. Gheusi et al. 1994; Cuadrado 2000; Colley and Parmentier 2012). Several authors have proposed that communication signals exhibiting sexual dimorphism would facilitate mate finding in animals where both sexes advertise (Langmore 1998; Emerson and Boyd 1999), and that monomorphic signals would facilitate communication within social groups competing for resources against other groups (West-Eberhard 1983).

General theoretical models have not yet elucidated if sexual monomorphism of signals is a by-product of genetic limitations for the expression of sexual signals in females (Kokko and Johnstone 2002). In contrast, phylogenetic analyses support the idea that the evolution of elaborated signals in females is not restricted by male genotypes (Ord and Stuart-Fox 2006; Odom et al. 2014). In consequence, mutual sexual selection has been proposed to account for the occurrence of sexual monomorphism (Langmore 1998; Prudic et al. 2011; Tobias et al. 2012; Atsumi et al. 2019). Accordingly, both sexes are expected to produce signals of roughly equal magnitude when the intensity of intra-sexual competition alternates between the sexes, i.e. when the sex that displays sexual signals and struggle for attracting the other sex switches over time (Kokko and Johnstone 2002). Non-territorial birds in which both sexes display sexual signals can be ranked along a dimorphism-monomorphism continuum, depending on whether only one or both sexes exert choice, respectively (Trail 1990).

In species exhibiting vocal interactions, female signalling may have several functions in addition to partner attraction, including territory defence from intruders, who may compete with the emitter for food, attempt to take over nest sites or engage in extra-pair copulations (Collins 2004; Tobias et al. 2012; Riebel et al. 2019). As such, the ecological and evolutionary processes underlying sexual signalling are similar for females and males when both sexes signal (Tobias et al. 2012; Riebel et al. 2019) and the effects of intra-sexual competition parallel those of competition between offspring within a

brood, as attracting mates by means of songs in the former assimilates to soliciting food using loud screams to attract the attention of parents in the latter (West-Eberhard 1983; Clutton-Brock and Huchard 2013). In diverse colonial species, contact calls allow recognition between parents and offspring within a crowd of conspecifics, and such cases also import a social dimension of signal evolution not dependent on a sexual context (Kondo and Watanabe 2009). In all cases where animals exchange social signals, individual signalling is a prerequisite for the emergence of social recognition (Gheusi et al. 1994; Bee 2016; Keen et al. 2016). Therefore, signals of social species are expected to be distinguishable at the level of individuals.

Signal recognition among sexual monomorphic conspecifics is not straightforwardly explained (Langmore and Bennett 1999) and requires additional information about the mating systems of the populations studied (Owens and Thompson 1994). For example, the operational sex ratio (OSR, i.e. the ratio between active males and receptive females ready to mate in a population at a given time) is a relevant population parameter to predict the intensity of intra-sexual competition (Emlen and Oring 1977). In theory, the sex with the lower parental investment will be the sex towards which OSR is biased (Owens and Thompson 1994; Kvarnemo and Ahnesjö 1996). Thus, when parental care is carried out by males, it is expected that more females than males are available for mating in the breeding population (Owens and Thompson 1994; Kvarnemo and Ahnesjö 1996; Clutton-Brock 2007). Male parental care is a condition of sexual role reversal (Owens and Thompson 1994), in which females typically struggle to be chosen and to receive parental care from males to their offspring (Goymann et al. 2004; Berglund et al. 2005).

OSR bias in a population can be caused by the sexual ratio at birth or hatching, and/or differences in development speed or in survival (Owens and Thompson 1994; Kokko and Monaghan 2001; Goymann et al. 2004; Kvarnemo et al. 2006). Temperature and the availability of food and nesting sites can also affect OSR and the reproductive potential of the sexes (Kvarnemo and Ahnesjö 1996; Hare and Simmons 2020). However, even though the raw numbers of males and females are similar within a given population, the OSR can be biased if individuals of each sex have different times out of the reproductive pool either by raising offspring or recovering energy from their last reproductive event (Kvarnemo and Ahnesjö 1996; Kokko and Johnstone 2002). As such, estimating OSR based on frequencies of males and females within a population does not necessarily reveal the readiness of

individuals to mate and eventually to compete (Kvarnemo and Ahnesjö 1996). In animals that base their reproductive behaviour on sound displays, recordings of vocalization rates are used as a proxy to estimate intra-sexual competition while attracting potential mates (Stoddard et al. 1988; Bosch and Márquez 1996; Goymann et al. 2004; Cui et al. 2010), but OSR is rarely analysed in vertebrates.

In anurans, from the over 7000 extant species, the percentage of taxa in which females vocalize is less than 1% (Emerson and Boyd 1999; Wells 2007; Köhler et al. 2017). Like the calls of male anurans, female calls can also have several functions related to reproduction (mate attraction and reciprocity to male calls), including nesting defence (Stewart and Rand 1991) and sexual mimicry to evaluate potential mates (Serrano and Penna 2018). In anuran species in which females produce calls, three calling patterns have been identified: (1) female advertisement calls are sexually dimorphic relative to males (Schlaepfer and Figueroa-Sandi 1998; Emerson and Boyd 1999; Goyes Vallejos et al. 2017), (2) females produce aggressive calls towards males during mating events (Cui et al. 2010; Preininger et al. 2016) or non-mating contexts (Capranica 1965; Stewart and Rand 1991); and (3) females produce sexually monomorphic signals that only differ depending on body size dissimilarities with those of males (Serrano and Penna 2018).

To further explore the structuring factors of the monomorphism-dimorphism continuum of sexual signalling in vertebrates, we used the southern Darwin's frog (*Rhinoderma darwinii*) as a model to analyse sexual dimorphism in a species with female vocalization. This frog is worldwide known due to its peculiar reproduction mode, as males brood the tadpoles inside their vocal sacs (i.e. neomelia; Jimenez de la Espada 1872). *Rhinoderma darwinii* individuals do not exhibit clear external sexual dimorphism (although males are easily recognizable when brooding offspring). However, females have a larger body size than males (Crump 2002; Valenzuela-Sánchez et al. 2014) and dorsal colouration can differ between sexes in some northern populations of the species (Bourke et al. 2011).

In the field, *R. darwinii* males usually call alone, in pairs or in small groups, positioned in moss mounds or herbaceous plants within the temperate forest (Crump 2002; Valenzuela-Sánchez et al. 2014). Vocal activity of *R. darwinii* is mainly diurnal, and its vocalization is restricted to the warmer months, from October to March, when breeding takes place (Penna and Veloso 1990; Crump 2002; Serrano et al. 2020). Although Busse (2003) reported that *R. darwinii* females produced single note calls during mating in captivity, the calling behaviour of females has been poorly studied (Serrano et al. 2020). To fill this knowledge gap, in this study, we compared the seasonal vocal activity and the structure of calls emitted by males and females of *R. darwinii* in a natural population from Southern Chile, to evaluate the hypothesis that the position

along the monomorphism-dimorphism continuum of sexual signals depends on the time fluctuation of sex experiencing intra-sexual competition. We explored factors that could explain differences in call attributes between sexes, in order to provide further empirical evidence supporting rarely tested hypothesis that the lack of dimorphism in sexual signals might evolve in systems experiencing temporal changing conditions of intra-sexual competition. In addition, we analyse the variation of these displays to estimate their potential for sexual recognition and intra-sexual competition. Furthermore, we evaluate the extent of sex reversal on vocal expression in a species having an exclusive paternal care behaviour.

Materials and methods

Study area and general procedures

Between October 2015 and February 2016, we conducted population monitoring and call recording in the private wild protected area Tantauco Park, located in Chiloé Island, Southern Chile (43° 21' S; 74° 6' W). A rectangular plot of 250 m² containing a local population of *R. darwinii* was monitored each month. During each month, we counted the total number of individual frogs found within the study plot in a search performed along three consecutive days (total effort = 12 person-hours per month). From November to February, when *R. darwinii* frogs were heard calling, we recorded individual calls during five days each month. Each captured frog was body size measured (snout-vent length, SVL), weighed and photographed for individual recognition using their ventral colouration patterns (Soto-Azat et al. 2013). Based on body size and morphological characteristics (i.e. coelomic enlargement indicative of internal eggs in females, presence of tadpoles in vocal sacs of pregnant males, and presence of two small openings on each side of the mouth floor under the tongue characteristic of vocal sacs of non-pregnant males), frogs were classified as males (pregnant and non-pregnant), females, juveniles or recently metamorphosed frogs. Previous studies have used these stages to analyse ecological aspects of *R. darwinii* populations (Crump 2002; Valenzuela-Sánchez et al. 2014, 2019). Recently metamorphosed frogs are very small individuals (< 10 mm SVL) still having tails and juveniles are individuals with a SVL shorter than 18.8 mm (as that was the smallest size observed in calling individuals in this population; see "Results"). In pregnant males, the number of tadpoles inside the vocal sac were counted externally as tadpoles moved. Our study involved focal animals in the field, and the sexual status of frogs was known after the recordings were obtained. After sampling, each frog was released at the exact site of capture. It was not possible to record data blindly because our study involved focal animals in the field.

Individual acoustic recordings

We recorded 32 individuals of *R. darwinii*. Of these, 11 were non-pregnant males, 11 pregnant males and 10 females. We recorded eight to 27 calls per individual (mean = 14.7 ± 7.8 SD) during daily recording sessions. The values of all recorded calls were averaged for each individual and subsequent analyses were performed using these averages as independent replicates. Some individuals were recorded more than once on different days. In total, 13 *R. darwinii* individuals (10 non-pregnant males, two pregnant males and one female) were recorded on two to four different days with intervals between recordings from 1 to 62 days. During these recording sessions, four to 33 calls were recorded. Recordings of repeated individuals were used to assess call individuality (see below). The recordings were conducted with a directional microphone Sennheiser ME-66 positioned at $\sim 90^\circ$ and at different distances from the subject, depending on the terrain topography (range, 18–68 cm) and connected to a digital recorder Tascam DR-100 set at a sampling rate of 44.1 kHz and 16-bit resolution. To measure the amplitude of the vocalizations, we registered the root mean square (RMS) sound pressure level (SPL re 20 μ Pa, C frequency weighting and fast time weighting) of the calls placing the microphone of a sound level meter (Extech 407780) adjacent to the tip of the directional microphone. The sound level meter was calibrated each day before recordings by introducing its microphone inside the opening of a sound calibrator (Brüel and Kjaer 4231), which delivers a continuous 1-kHz tone at 94 dB SPL RMS.

Acoustic analysis

We analysed seven call parameters of advertisement calls by manually selecting notes and calls from oscillograms and spectrograms, using the software Raven Pro 64 v.1.4, employing a Hamming window and 256-sample fast Fourier transform (FFT). These included the following: (1) number of notes per call, (2) note duration, (3) call duration, (4) dominant frequency, (5) average entropy, (6) aggregate entropy and (7) call repetition rate. Note duration, dominant frequency and the two measures of entropy were measured for all the notes recorded. To standardize the measurements of call duration, this parameter was obtained from calls most frequently produced (i.e. four note calls; see Fig. S1). Average and aggregate entropy were measured as a proxy of spectral complexity related to the non-linear phenomena contained in advertisement calls of this species (Serrano et al. 2020). We calculated call repetition rate as the number of calls recorded during the 5 min following the first call produced.

Statistical analyses

Prior to performing analyses, normality criteria were verified for all the variables using the graphical method of quantile-quantile plot (Crawley 2013). Simple Pearson correlations were used to explore the association between acoustic variables of the calls with morphometric (SVL and weight) variables, and to evaluate the influence of distance between microphone and individuals on the recorded variability of SPL amplitude. Additionally, Spearman correlations were used to evaluate the association between acoustic properties of the calls of pregnant males and the number of brooded tadpoles. Acoustic features and body size measures were compared using analysis of variance (ANOVA) to determine whether call properties varied among individuals of different sexes. The Tukey's honest significant difference method was used to estimate post hoc analyses in those cases where ANOVA yielded significant results. To identify whether sexual differences in acoustic variables depended on body size differences among sexes, datasets were re-analysed correcting the data by SVL (following Platz and Forester 1988) for those variables for which significant relationships were detected (see "Results") and ANOVAs were repeated afterwards. Due to technical problems while in the field, we were not able to record air temperature during the recording of calls, and, therefore, unable to perform a temperature correction using a similar procedure as with body size. However, a previous study reported that temperature is related only to call duration in non-pregnant males of *R. darwinii* (Serrano et al. 2020). We performed all statistical analyses using R (R Development Core Team 2019). When required, Box Cox transformations were performed with the R's package MASS (Venables and Ripley 2002) to fulfil normality assumptions of ANOVA. The ANOVAs were implemented in the R's package car (Fox and Weisberg 2019). We estimated the chances to discriminate advertisement calls of males and females using a discriminant function analysis (DFA) performed for five acoustic variables (note duration, dominant frequency, aggregated entropy, call rate and sound pressure level). Such variables were selected after examining collinearity between all the variables measured from advertisement calls to reduce the set of variables and improve the classification power of the DFA method (Naes and Mevik 2001). However, call repetition rate values were obtained from all call types (i.e. calls of one to six notes) emitted by all the individuals recorded. Prior to performing DFA, variables were centred and scaled for standardization purposes (Crawley 2013). To evaluate the contribution of the different variables to the sexual distinctiveness model, we conducted a stepwise forward selection of variables using the Wilk's Lambda criterion (Karson and Martell 1980). To

do this, an overall model was defined first, to establish the contribution of each variable to discriminate the groups. Subsequently a differential resampling was applied to evaluate each independent variable including it into the model and then taking it out, calculating a Wilk's Lambda statistic. A ranking of relative importance is thereafter completed and the allocation of the largest value of Wilk's Lambda to a variable is interpreted as a sign of its importance for discrimination, having the next largest value of Wilk's Lambda as the following most significant variable (Karson and Martell 1980). The significance of the change in the lambda statistic was evaluated with a F test, and if the F value was greater than the critical value, the variable was kept in the model, in order to detect which variable support differentially the significance of lambda values (Karson and Martell 1980). The R's packages MASS (Venables and Ripley 2002) and klaR (Roever et al. 2020) were used for DFA and Wilk's Lambda statistical analyses, respectively.

To quantify acoustic repetition attributable to individual calls we estimated the intra-class correlation coefficient (ICC) from all the individuals recorded (Wolak et al. 2012). This repeatability index can reach values from 0 to 1, where 0 indicates lack of repeatability and 1 indicates high repeatability (Cicchetti 1994; Wolak et al. 2012). In psychological studies, it is considered that when the value of the ICC is below 0.4 the repetitiveness is low, values between 0.4 and 0.6 are considered reasonable, between 0.6 and 0.75 are good and above 0.75 the recurrence is excellent (Cicchetti 1994). In both psychological and behavioural studies, ICC values tend to perform similarly with low values and wide dispersion (Wolak et al. 2012). Repeatability estimation of ICC was supported by the calculation of confidence intervals, number of measurements expected, and variance associated with these estimates, following Wolak et al. (2012). To estimate repeatability calculations, we applied the R's package ICC (Wolak et al. 2012).

Data availability

The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

Results

Raw counts and calling behaviour

We made 306 captures of 156 individuals of *R. darwinii* (56 non-pregnant males, 36 pregnant males, 26 females, 33 juveniles, and 5 recently metamorphosed frogs). Forty-four percent of the individuals were recaptured at least once (range = 2–9 captures, mean = 3.3 ± 1.6 SD). We found a gradual

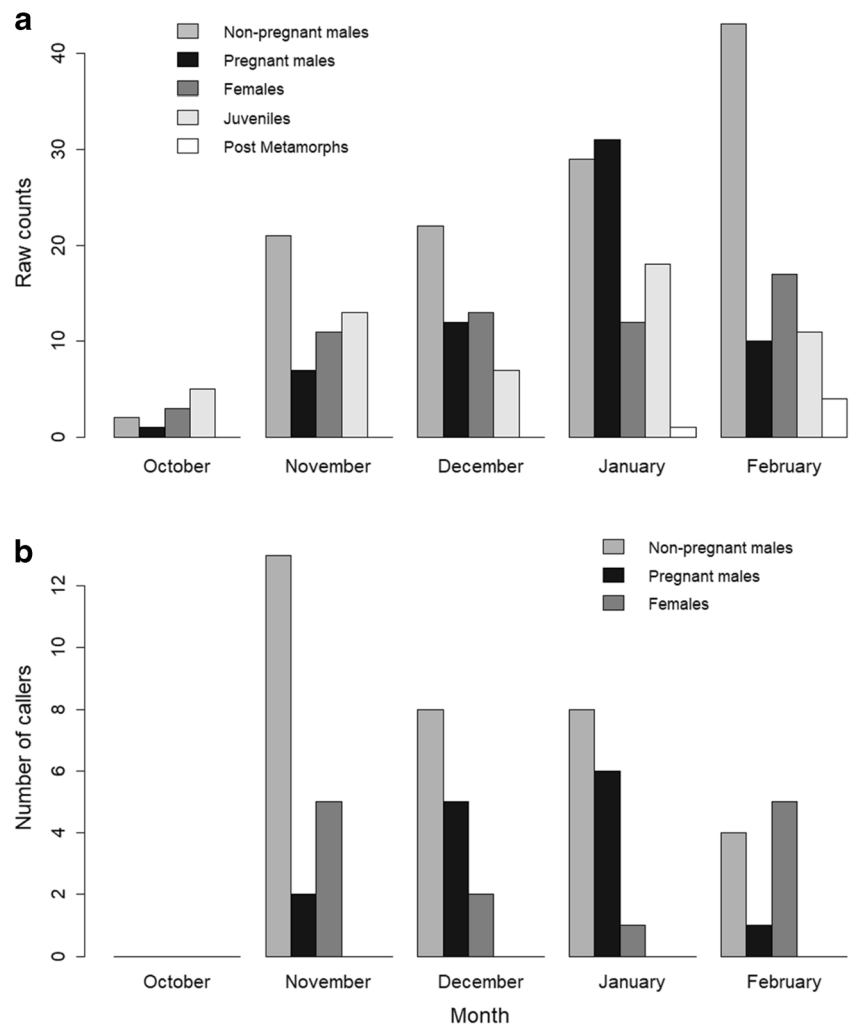
increase in the number of captured non-pregnant male and female frogs from October to February, but a decrease of pregnant males and juveniles in February relative to January (Fig. 1a). In non-pregnant males, the raw counts of frogs (callers and non-callers) and the proportion of callers showed an opposite pattern across the season: while the raw counts of non-pregnant males increased, the proportion of non-pregnant calling decreased. In pregnant males, the maximum raw count occurred in January, decreasing in February, and the proportion of callers increased from November to December, reaching its maximum in this month. A different pattern was observed in females. While the raw counts of females slightly increased along the season, the proportion of female callers peaked at the beginning (November) and the end (February) of the season (Fig. 1b).

When vocalizing, frogs produced advertisement calls spontaneously for periods of 3 to 20 min with irregular silent intervals between calls. In general, individuals were observed vocalizing during up to three continuous days (see below). No aggressive calls or physical attacks were observed for individuals of any sex during this study.

Sexual differences and call distinctiveness

Pregnant males carried from one to nine tadpoles in their vocal sacs (median = 3; Fig. S2), body size of pregnant males was not related to the number of brooded tadpoles ($r = 0.24$; $df = 34$; $p = 0.177$), but male body mass and number of brooded tadpoles were positively related ($r = 0.60$; $df = 34$; $p < 0.001$). We found that sexes differed in body size and body mass (Table 1). Females had a larger body size than pregnant and non-pregnant males (Tukey's honest significant difference, $p < 0.05$), and for body mass, females were the heaviest and non-pregnant males the lightest (Tukey's honest significant difference, $p < 0.05$ for all comparisons). In pregnant males and females, we did not find differences in body size between calling and non-calling individuals (Mann-Whitney U test = 243, $p = 0.859$; Mann-Whitney U test = 201, $p = 0.837$, respectively). In contrast, in non-pregnant males, callers had a longer body size than non-callers (Mann-Whitney U test = 521.5, $p < 0.001$). To better understand the reproductive performance of the two sexual types of males, we analysed the changes in body size of those males that switched and those that maintained their sexual status of pregnancy throughout monthly recaptures (see Supplementary information). Overall, we found no differences in mean body size between pregnant and non-pregnant males at the beginning of the study and those that switched their reproductive status (Fig. S3). However, males that switched their reproductive status across the season from non-pregnant to pregnant, had a higher increase in body size than males that remained pregnant throughout the complete season (Fig. S4).

Fig. 1 Individual raw counts (a) and number of callers (b) for the different sexes and age ranges of *Rhinoderma darwinii* per month along the period of study (October 2015 to February 2016) on Chiloé Island



Among the 118 adults of *R. darwinii* captured in the population studied, only 27% (32 individuals) were recorded calling. This corresponds to 20% of non-pregnant males, 31% of pregnant males and 38% of females observed. The calls of males and females were not distinguishable by the researchers in the field.

In males and females, the most frequently produced call was the one composed by four notes (non-pregnant males = 44%, pregnant males = 64% and females = 58% of the calls; Fig. S1).

Because of their high prevalence, we compared call duration between sexes using only the four-note calls. Examples of

Table 1 Average \pm standard deviation of body size and acoustic features for males and females of *Rhinoderma darwinii*

Acoustic variable	Non-pregnant males (N = 11)	Pregnant males (N = 11)	Females (N = 10)	F value
Snout-vent length (mm)	21.99 \pm 0.77	22.17 \pm 0.64	23.22 \pm 0.97	6.583**
Mass (g)	0.79 \pm 0.07	0.96 \pm 0.08	1.06 \pm 0.10	25.431***
Note duration (ms)	0.153 \pm 0.03	0.165 \pm 0.02	0.191 \pm 0.03	4.970*
Call duration (s)	1.55 \pm 0.17	1.63 \pm 0.16	1.87 \pm 0.20	8.122**
Dominant frequency (Hz)	3712 \pm 229	3651 \pm 309	3391 \pm 246	3.998*
Aggregated entropy	2.01 \pm 0.47	2.05 \pm 0.61	2.29 \pm 0.54	0.757
Average entropy	1.96 \pm 0.40	1.95 \pm 0.35	2.02 \pm 0.28	0.097
Call rate (calls/min)	1.45 \pm 0.52	1.25 \pm 0.61	1.71 \pm 0.44	1.819
Sound pressure level (dB)	62.77 \pm 4.59	62.54 \pm 5.42	66.08 \pm 4.78	1.374

F values indicate ANOVA comparisons between populations

* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ —significance levels

advertisement calls from the sexes are shown in Fig. 2. The high proportion of chaos shown in Fig. 2a is not a unique characteristic of non-pregnant male calls, but it is in this group where it occurs most frequently. Females had longer note and call durations and lower dominant frequency than non-pregnant and pregnant males (Table 1; Fig. 3). No statistical differences were found among males and females in aggregated entropy, average entropy, call rate and call SPL. When we re-analysed these datasets correcting by SVL, analyses showed that all the acoustic variables did not differ significantly between sexes (ANOVA tests, call duration, $F = 3.226$, $p = 0.057$; note duration, $F = 2.618$, $p = 0.091$; dominant frequency, $F = 2.467$, $p = 0.104$), which indicates that differences among males and females in these acoustic variables are likely related to body size differences between the sexes.

Comparisons of call amplitudes among individuals were not affected by the distance from the focal individual to the microphone, as we did not find a significant correlation between this measure and recording distance ($n = 18$; $r = -0.13$; $df = 16$; $p = 0.612$). The lack of correlation between these variables is most likely a result of the topographical complexity of the microenvironment in which *R. darwinii* calls, comprising mounds, roots and dense understory vegetation (Penna and Moreno-Gómez 2015). This lack of relationship validates our comparisons of individual calls registered at distances ranging from 18 to 68 cm. We found a negative relationship between the number of tadpoles brooded and call amplitude in pregnant males ($r = -0.79$; $df = 6$; $p = 0.019$), indicating that males brooding less tadpoles called louder. Although the median number of tadpoles counted in calling and non-calling pregnant males was the same (i.e. 4 tadpoles), the range of this parameter was wider in non-calling (range = 2–9 tadpoles) than in calling pregnant males (range = 2–5 tadpoles).

The data used for the DFA included only four-notes calls, which were produced by 22 individuals (eight non-pregnant males, seven pregnant males and seven females). Call duration and average entropy were omitted from the DFA given their collinearity with note duration and aggregated entropy,

respectively. All the five acoustic variables incorporated in DFA were chosen due to their contribution to the whole model; however, only dominant frequency was statistically significant in the differential model (differential F), which means that only this variable was relevant after the re-sampling procedures of Wilk's Lambda criterion and therefore the distinctive model was composed only by this variable (Table 2). Two linear discriminant vectors (LD) were projected explaining 96% (LD1) and 4% (LD2) of the data (Fig. 4). The graphic distinctiveness between males and females was more noticeable than between pregnant and non-pregnant males, as females were separated from both types of males by LD1 and LD2. A priori probabilities of discrimination were 35, 35 and 30% for non-pregnant males, pregnant males and females, respectively. With these probabilities, 90.9% of the data were correctly assigned to females and the two types of males, and only the mean values of calls from one female and one non-pregnant male were assigned erroneously to pregnant males.

Individual repeatability of calls

Of the 13 individuals recorded more than once on different days, 12 were males and only one of them changed its breeding status across the recording period (from non-pregnant to pregnant; Fig. 5). Note duration, call duration, aggregated entropy, average entropy and SPL showed high individual repetitiveness (values ranged between 0.61 and 0.67; Cicchetti 1994), while repetitiveness was moderate for dominant frequency (0.5) and poor for call rate (0.19). In Table 3, we show a summary of the variables from repeatability calculations.

Discussion

Our study provides evidence indicating that the calls of *R. darwinii* are closer to the monomorphic end of the continuum of sexual dimorphism. We found that female calls are

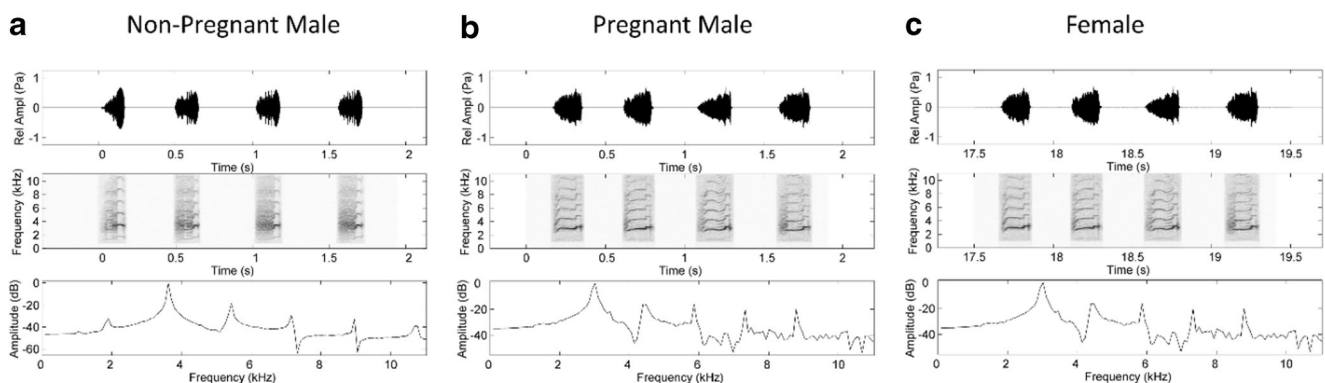


Fig. 2 Oscillograms, spectrograms and power spectra of four-note advertisement calls of a non-pregnant male (a), a pregnant male (b) and a female (c) of *Rhinoderma darwinii* recorded on Chiloé island, Chile.

Sample rate was 44.1 kHz for all the recordings. Snout-vent-length of (a), (b) and (c) were 21.3, 20.9 and 23.6 mm, respectively. Air temperatures during recordings were 14.6, 21.6 and 16.2 °C, respectively

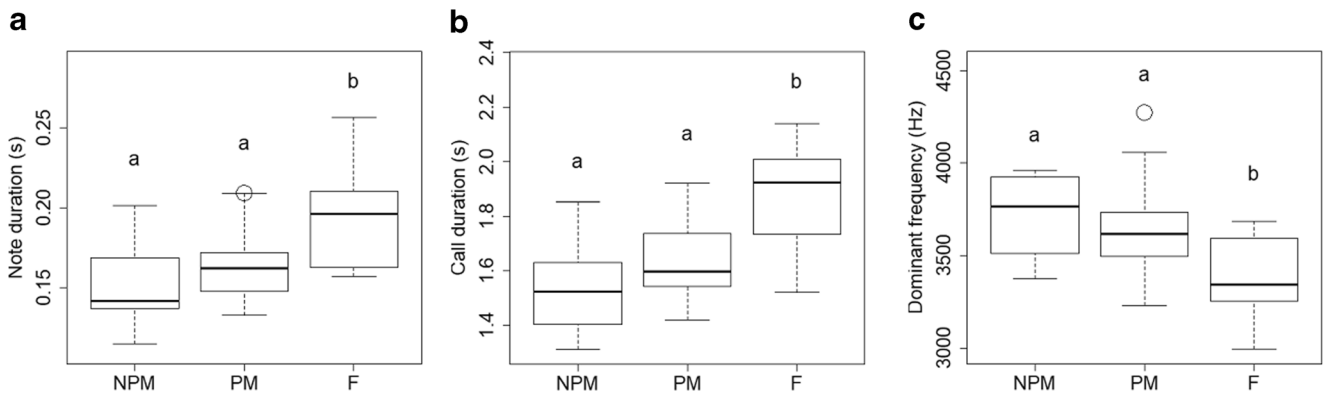


Fig. 3 Note duration (a), call duration (b) and dominant frequency (c) for males and females of *Rhinoderma darwinii*. Boxes correspond to the first and third quartiles (lower and top border of the box) and the median (thick horizontal line within the box). Whiskers extending from the top and bottom borders of the box indicate the 90th and 10th percentiles. The

individual points outside the whisker boundaries indicate outlier values. Lowercase letters (a, b) indicate significant differences between sexes in post hoc analyses ($p < 0.05$). Abbreviations: NPM, non-pregnant males; PM, pregnant males; F, females

similar in their spectral and temporal characteristics to those of males (pregnant and non-pregnant). The vocal behaviour of *R. darwinii* exhibits low dimorphism in call structure and different patterns of vocal activity between the sexes along the breeding season. As displaying advertisement calls is related to reproductive readiness in anurans, we interpreted such alternated vocal patterns in terms of OSRs. Vocal exhibition patterns showed intra- and inter-sexual selection could be limited due to such low levels of sexual signal dimorphism found. Overall, our data opens the possibility that other mechanisms such as social selection are necessary to understand this communication pattern.

In this study, the number of *R. darwinii* individuals captured and the proportion of those individuals displaying vocal signals varies throughout the season, and this temporal variation was sex specific. In non-pregnant males, the number of individuals increased but the number of callers diminished along the reproductive season. In pregnant males, the number of individuals increased from the beginning of the season,

reaching a peak in January and then decreased in February. For females, the proportion of individuals increased from the beginning to the end of the season, but their vocal activity of females peaked inversely relative to that of pregnant males, reaching maxima at the beginning and end of the breeding season. Interestingly, throughout the four months of breeding season, the number of males available for mating is not limited at any time (Fig. 1b). Indeed, vocal activity of males exhibits three scenarios of male availability for reproduction, the first would consider both non-pregnant and pregnant males (as it is unknown if pregnant males can get additional pairings) resulting in male-biased OSR during the entire study period. A second scenario considering only non-pregnant males results in a similar OSR male-biased. However, the third scenario considering only pregnant males suggest a changing scenario from being female-biased at the beginning and end of the season, to male-biased between December and January. This last scenario is the most likely since it is supported by the evidence that only 40% of males are taking care of tadpoles,

Table 2 Relative importance of acoustic variables for males and females distinctiveness in *Rhinoderma darwinii*

	LD1	LD2	Wilk's lambda	Overall F^a	Differential F^b
Dominant frequency	0.952	− 0.060	0.625	5.690*	5.690*
Call rate	− 0.513	− 0.393	0.474	4.076**	2.882
Note duration	− 0.565	− 0.095	0.420	3.080*	1.093
Aggregated entropy	− 0.451	− 0.130	0.388	2.419*	0.646
Sound pressure level	− 0.385	0.737	0.349	2.080	0.852
Eigenvalues	4.010	0.786			
Explained %	96.3%	3.7%			

Linear discriminant coefficients (LD), eigenvalues, and explained variance percentages of discriminant function analysis

* $p < 0.05$, ** $p < 0.01$ —significance levels for F values

^a The contribution of the acoustic variable for the overall model

^b The contrast comparing the model including and excluding the corresponding acoustic variable

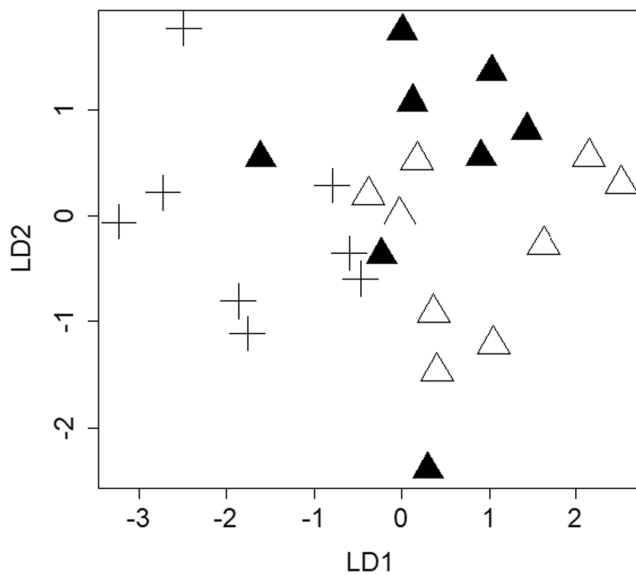


Fig. 4 Linear discrimination functions (LD1 and LD2) representing males and females' distinctiveness in *Rhinoderma darwinii* advertisement calls. Symbols identify non-pregnant males (empty triangles), pregnant males (filled triangles) and females (plus sign)

and therefore, they are presumably monopolizing paternity of offspring within the population, as what occurs in other sexual role-reversed species (Møller and Birkhead 1993; Kvamemo et al. 2006).

So, as pregnant males and females of *R. darwinii* call preferentially during different periods of time along the breeding season, it is expected that mate choice is exerted by each sex with different intensity throughout the reproductive season,

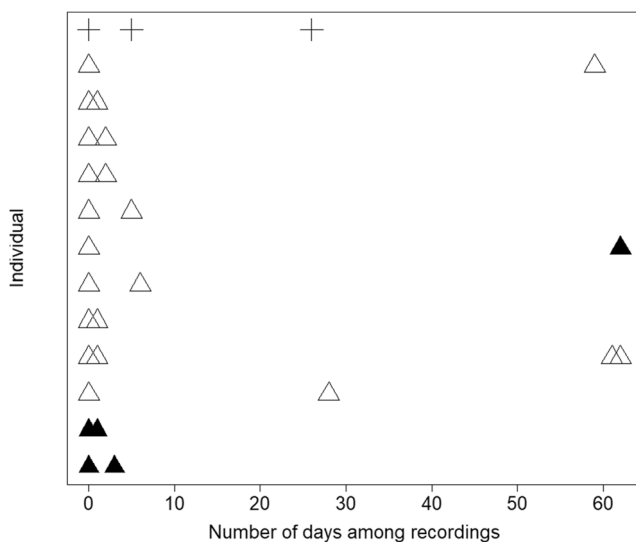


Fig. 5 Intervals between days in which calls of 13 individuals of *Rhinoderma darwinii* (10 non-pregnant males, two pregnant males and one female) were recorded on two to four opportunities. Symbols identify individuals of males and females: empty triangles, non-pregnant males; filled triangles, pregnant males; plus sign, females. Only one non-pregnant male was recorded on a second opportunity when it was pregnant

causing greater intra-sexual competition when the corresponding sex is actively calling (Clutton-Brock 2007). However, our observations indicate that, in this population, few males call actively on the same day and that it is unlikely to hear more than one female calling on the same day. In *R. darwinii*, females have restricted home ranges relative to males around moss mounds in the humid forest, and according to our observations and previous studies (Valenzuela-Sánchez et al. 2014), neither male nor female *R. darwinii* individuals engage in aggressive behaviour. Therefore, the intra-sexual competition in this species is unlikely to be very intense.

Cases in which sexual selection pressures alternate between sexes along the breeding period have been observed to occur as a result of temporal fluctuations of each sex for recovering after mating (Vincent et al. 1994; Forsgren et al. 2004; Clutton-Brock 2007; Reichard et al. 2007; Kasumovic et al. 2008). However, our study is the first report implying a potential fluctuation in sexual competition paralleling an alternation in vocal display of the sexes and potential OSR fluctuations. Switches in OSRs between female- and male-biased periods could restrict competition between males if mortality for competing for mates is higher than from caring (Kokko and Jennions 2008). In fact, males of *R. darwinii* could care for tadpoles and be racing by sperm competition instead of competing (vocally) to obtain mates, as occurs in other species with sex role reversal (Emlen et al. 1998; Goymann et al. 2004). Regarding females, our data suggest that *R. darwinii* females could have more than one reproductive event per season as they are more vocally active at the beginning and end of the season, which could imply that females are sequentially ready for mating. This mating behaviour leads females to ensure their reproductive success in other species with reversible sex roles (Hare and Simmons 2020). Such a female-biased scenario with few males available to reproduce does not necessarily imply higher intra-sexual competition in females (Vincent et al. 1994).

Male and female advertisement calls in this study differed in their dominant frequency, note duration and call duration. Call differences between sexes can be attributed to differences in body size, as reported in non-human primates (Ey et al. 2007), birds (Geberzahn et al. 2009), fishes (Colleye and Parmentier 2012) and anurans (Serrano and Penna 2018). Our analyses showed that sex differences in the above-mentioned acoustic variables in *R. darwinii* were only due to differences in body size, as females were larger than males. This is a common finding in amphibians. Indeed, body size in anuran females is expected to be under strong selection as larger females produce more eggs and reach sizes that optimize amplexic holding by males (Shine 1979; Woolbright 1985; Arak 1988). These differences may also reflect differences in growth rates (Monnet and Cherry 2002; Kupfer 2007) or longevity between sexes (Lemaitre et al. 2020). In *R. darwinii*, differences in body size between sexes could

Table 3 Intra-class correlation (ICC) values for acoustic features of individuals of *Rhinoderma darwinii* from Chiloé Island, Chile

Acoustic feature	<i>n</i>	ICC	CI 95%	<i>k</i>	VarW	VarA
Note duration	30	0.62	0.51–0.75	80.29	0.0006	0.0009
Call duration	28	0.66	0.54–0.79	11.71	0.021	0.040
Dominant frequency	30	0.50	0.39–0.65	80.29	66060.1	66590.3
Aggregated entropy	30	0.63	0.52–0.76	80.29	0.190	0.326
Average entropy	30	0.61	0.50–0.74	80.29	0.089	0.140
Call rate	30	0.19	– 0.42 to 0.57	1.55	0.295	0.069
Sound pressure level	24	0.67	0.54–0.80	14.59	8.927	17.940

Estimations of confidence intervals (CI 95%), number of expected measurements (*k*), variance within (VarW) and among (VarA) individuals (*n*) are shown

result from diverse selective pressures like these, and call spectral differences may entail a by-product of such processes.

In *R. darwinii*, the monomorphic characteristics of the call could promote a social function of this signal, as has been proposed to occur because of social selection (West-Eberhard 1983). Indeed, monomorphic signals can mediate social cooperation among individuals within a group (Gheusi et al. 1994; Roughgarden 2012). In anurans, acoustic signals are known to modulate social interactions mostly in males (Bee 2016). Yet, female anurans can aggregate in lek choruses (Goyes Vallejos et al. 2017), and in some cases exhibit aggressive calls during social interactions (Capranica 1965; Preininger et al. 2016) and nesting defense (Stewart and Rand 1991). The social importance of female calls in our model species, however, could be related to other functions as described in several animal species. For instance, female signals can indicate social status (Keen et al. 2016), maintain social control of resources (Lamba et al. 2008; Bang and Gadagkar 2012; Colleye and Parmentier 2012), coordinate collective displacements (Couzin and Krause 2003) or maintain species isolation (Atsumi et al. 2019).

Behaviours of male and female anurans is conducted by sexual hormones that feedback from the acoustic social environment in which they are (Emerson and Boyd 1999; Burmeister and Wilczynski 2000; Lea et al. 2001; Lynch and Wilczynski 2006). As such, vocalization by both sexes of *R. darwinii* could act to maintain breeding activity of natural populations providing effective sexual stimulation of local individuals of both sexes. Further exploration of the social relevance of male and female calls in *R. darwinii* could provide important insights on the evolution of monomorphic signals in animals.

Individuality is a requirement for social recognition (Gheusi et al. 1994; Bee 2016). Our results yield a high individual repeatability of advertisement calls in *R. darwinii*, and Serrano et al. (2020) also showed that advertisement calls of this species are highly individual. Adults of *R. darwinii* maintain a restricted home range for many years or perhaps throughout their lifetime (Valenzuela-Sánchez et al. 2014,

2019). The individual call stability suggests that peculiar features of vocalizations are relevant to sustain prolonged social interactions among permanent neighbours, as reported in other sound-communicating species (Sheehan and Bergman 2016). According to our results, it is possible that vocal interaction in *R. darwinii* facilitates the recognition of neighboring individuals and subsequent spacing.

Unexpectedly, *R. darwinii* males remain calling after they become pregnant. In these males, the number of tadpoles brooded affects the amplitude of their advertisement calls. As the vocal sac operates as a powerful resonator in anurans (Rand and Dudley 1993), a larger number of brooded larvae should interfere with the efficiency of call emission. Such a relationship raises the question of whether pregnancy status could be perceived by potential mates through the advertisement calls of pregnant males. However, calls of pregnant and non-pregnant males did not show amplitude differences. It is in concordance with studies on two midwife toads, *Alytes cisternasii* and *A. obstetricans*, showing no differences between the calls of males carrying and not carrying eggs (Márquez 1990). In *A. obstetricans* a relationship between body size and the number of eggs carried has been described in one population (Raxworthy 1990) but neither significant relationships of this kind nor differences between males that carry and do not carry eggs, have been reported in subsequent studies in midwife toads (Márquez 1993; Bush 1996; Lea et al. 2003). However, parental care carried out by *Alytes* males does not compromise the vocal apparatus directly as in *R. darwinii*, and thus the limited influence of pregnancy on the call structure of the South American species is noteworthy.

It is unclear whether all males of *R. darwinii* observed calling in the population are indeed reproductive adults. The dispersion in non-pregnant males' repertoire, ranging from calls with three to six notes, contrasts with the most frequent four-notes calls of pregnant males and females, suggesting that non-pregnant males have not reached a stereotyped pattern in their call repertoire. In anurans, sexually immature males have been found vocalizing within choruses composed

primarily by mature males (Goldberg et al. 2016; ten Hagen et al. 2016). In the South American hyloid frog *Pseudis minuta*, juvenile testes exhibit spermatogenesis up to secondary spermatid stage. In this species, frogs with juvenile characteristics emit calls with lower number of pulses and higher frequency calls than larger mature adults (Goldberg et al. 2016), which suggests that vocal activity can occur before sexual maturity. In *R. darwinii*, it is unclear whether non-pregnant males may include immature males. In a previous study in non-pregnant males of *R. darwinii*, we found that smaller individuals have calls with higher dominant frequencies and larger proportion of non-linear phenomena relative to larger individuals (Serrano et al. 2020), which suggests that *R. darwinii* individuals start to vocalize before reaching sexual maturity, as a large proportion of non-pregnant males remains without caring for tadpoles throughout the reproductive season. To better understand if the series of changes that *R. darwinii* can undergo acoustically (e.g. amount of chaos, repertoire size, call intensity) depends on age and pregnancy or if these modifications in calls occur based on the social context, requires a systematic monitoring of the ontogeny of vocal behaviour and playback studies.

Rhinoderma darwinii illustrates a quite peculiar mode of female calling in anurans, as vocalizations have a monomorphic structure, a condition different from other anurans that also exhibit sexual role reversal, but produce sexual dimorphic vocal signals (Emerson and Boyd 1999; Preininger et al. 2016). Unexpectedly, given that few males carry out parental care and are the ones who presumably monopolize most of the reproductive events in *R. darwinii*, our data showed that OSR calculated with pregnant males switched in this species from female- to male-biased during the breeding season. These changes in OSR would not affect brood care performed exclusively by males in *R. darwinii*, but can affect intra-sexual competition and mate choice (Hare and Simmons 2020). According to our data, vocal exhibition fluctuates between males and females in *R. darwinii* throughout the season. In various wildlife examples, fluctuations in OSR bias are associated with corresponding switches in the choosing sex (e.g. Gwynne and Simmons 1990; Kvarnemo 1994; Vincent et al. 1994) that may also favour signal monomorphism. Furthermore, we never observed intra-sexual aggressive behaviour in females nor more than one female calling on the same day, suggesting that females do not interact vocally with each other. This suggests that female vocalization is related to male attraction rather than to female-female competition.

Our study shows a new instance of monomorphic signals in an anuran with sexual role reversal, contributing a contrasting condition relative to sexual dimorphism models previously reported in anurans (Emerson and Boyd 1999; Preininger et al. 2016; Serrano and Penna 2018) and birds (Trail 1990; Tobias et al. 2012; Riebel et al. 2019). In *R. darwinii*, seasonal shifts in vocal displays occur concomitantly with a sexual

monomorphic pattern of acoustic signals, suggesting that the strength of intra-sexual selection may fluctuate paralleling the intensity of vocal activity. Efforts to gain insights on the reproductive system and natural history of this species are needed to better understand its behavioural ecology. Overall, the peculiar characteristics of the breeding behaviour of this species lead to explore outstanding questions related to the mechanisms for recognizing poorly differentiated signals, the evolution of male pregnancy and sexual role reversal, among many others.

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Authors' contributions JMS designed the study, collected and analysed the data and wrote manuscript drafts. MP contributed to the experimental design, data collection and manuscript writing. CA and MAM facilitated fieldwork, funding and manuscript writing. AV-S contributed to the manuscript writing.

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Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflict of interests.

Ethics approval This study was carried out in accordance with the Chilean law (Servicio Agrícola y Ganadero permit N° 9822/2015). The Ethics Committee of the Faculty of Sciences of the University of Chile approved the guidelines followed to carry out this study. All applicable international, national, and/or institutional guidelines for the use of animals were followed.

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Electronic Supplementary Material

Behavioral Ecology and Sociobiology

Monomorphic call structure and dimorphic vocal phenology in a sex-role reversed frog

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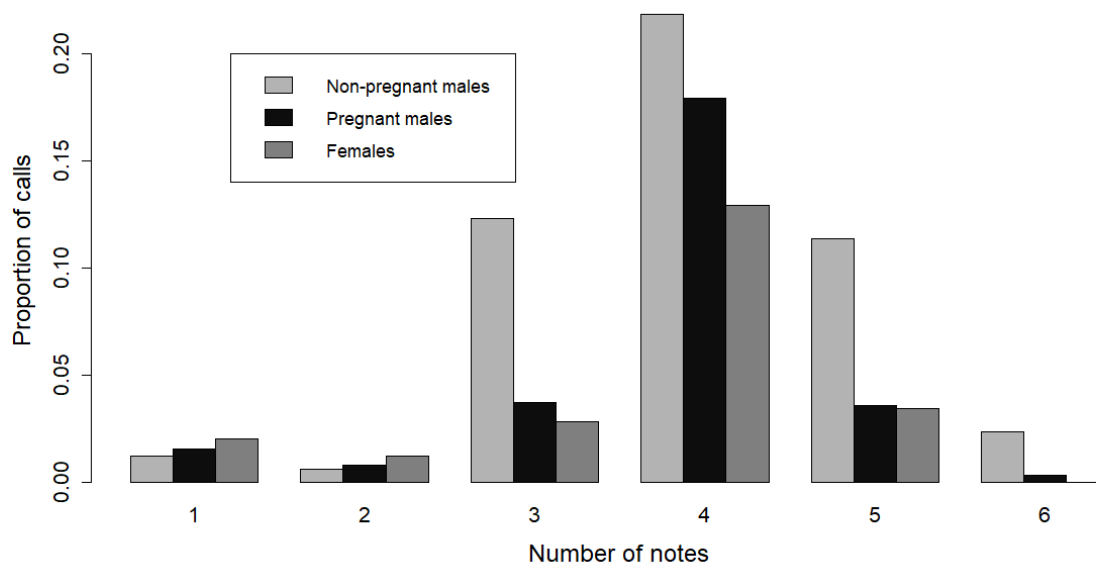


Figure S1 Number of notes per call in (non-pregnant and pregnant) males and females of *Rhinoderma darwinii*. To distinguish whether the types of advertisement call (in terms of the number of notes they contain) vary between sexes, the number of notes per call was analysed apart and the G test was used to compare it among males and females, yielding

significant differences ($G=44.10$; $df=8$; $p<0.001$). Because the G-test is sensitive to cells having no data, and no females produced calls having 6-note calls, we omitted these values from the comparison to perform this test. To identify significant differences between groups, we performed a pairwise comparison and found that the call repertoire of non-pregnant males differed from pregnant males ($p<0.001$) and females ($p<0.001$) but the call repertoire of pregnant males and females did not differ ($p=0.44$).

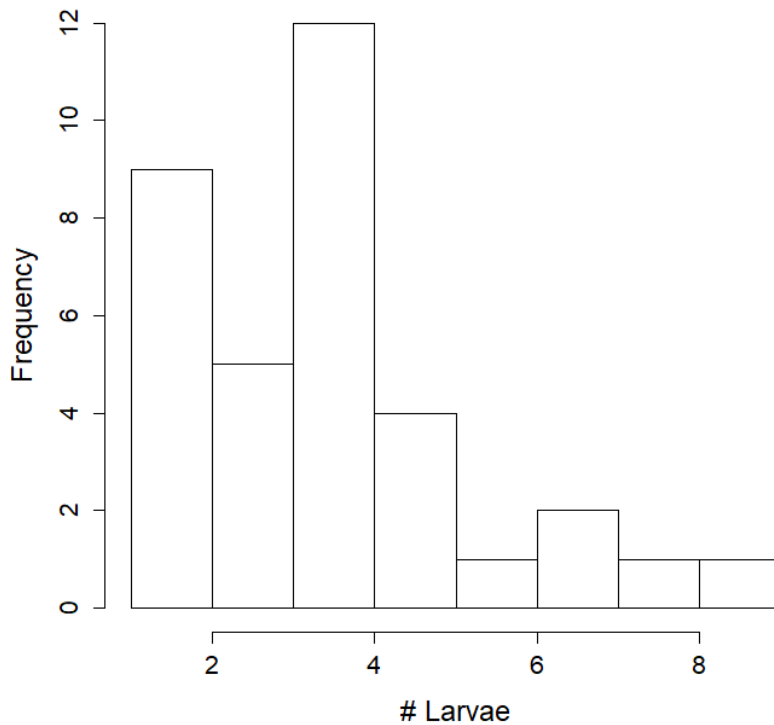


Figure S2 Frequency distribution of tadpoles counted between October 2015 and February 2016 in 35 pregnant males. For individuals with repeated counts, only the maximum number of tadpoles counted was considered.

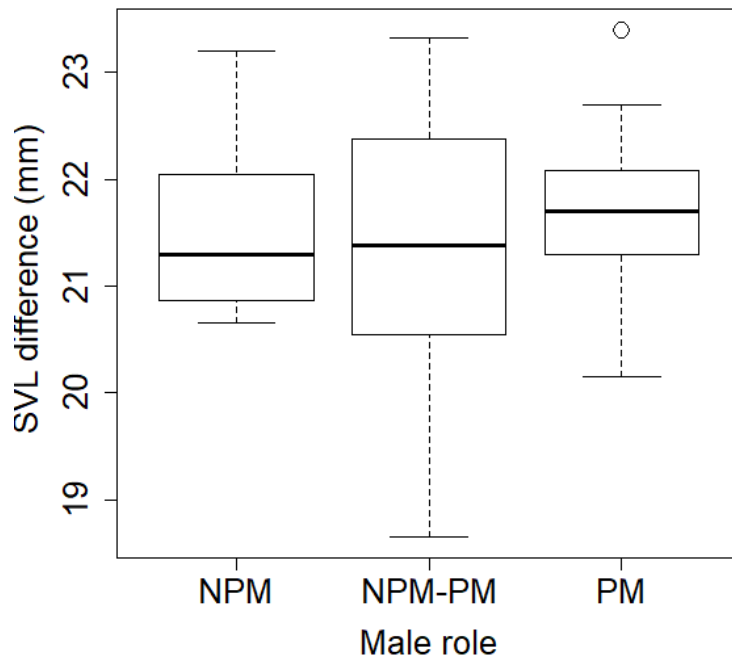


Figure S3 To determine if body size of males that maintain their reproductive status differ from those that change their status throughout the months of the study, we compared the difference in size of males i.e., males that remained non-pregnant (NPM), males that switched from non-pregnant to pregnant (NPM-PM) and males remaining pregnant or releasing toadlets (PM) at the end of February. We found that males of these three groups had similar initial mean body size ($F=0.491$; $p=0.615$).

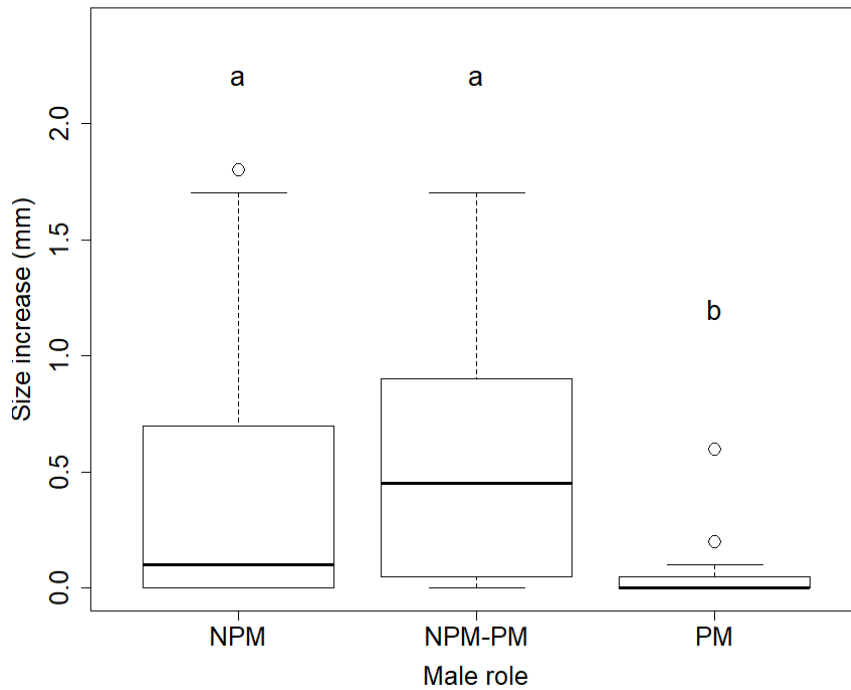


Figure S4 Size increments along the period of study in males that did not change their status of non-pregnant (NPM), males that changed from NPM to pregnant males (NPM-PM) and males that remained pregnant (PM). Pregnant males grew less relative to NPM-PM and NPM ($F=5.401$; $p=0.008$). Size difference along the period of study in those males that did not change their status of non-pregnant (NPM), males that changed from NPM to pregnant males (NPM-PM) and males that remained pregnant (PM).